IDENTIFICATION OF SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING TECHNOLOGY

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTOLOGY

MAY 2018

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"IDENTIFICATION OF SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. RAMYA ARUN, M.D.S.**, Reader, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

Dr. Anisha Deborah Jeyakumar
Post Graduate Student
Department of Periodontology
Ragas Dental College & Hospital,
Chennai.

Date: 25.01.2018

Place: Chennai

CERTIFICATE

This is to certify that this dissertation titled **"IDENTIFICATION OF SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide record of work done by **Dr. Anisha Deborah Jeyakumar** under my guidance during the study period 2015-2018.

This dissertation is submitted to **THE TAMILNADU Dr.MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

RAGAS DENTAL COLLEGE Chennai

Guide:

Dr. Ramya Arun, M.D.S.,
Reader,
Department of Periodontology,
Ragas Dental College & Hospital,
Chennai.

Department of Periodontics,
Ragas Dental College & Hospital
2/102, ECR, Chennai-119

Head of the Department:

25/1/18

Dr. K. V. Arun, M.D.S.,
Professor,
Department of Periodontology,
Ragas Dental College & Hospital
Chennai.

Dr.K.V.ARUN MDS
Head of the Department
Department of Periodontics
Ragas Dental College and Hospital
Chennai - 600 119.

Dr. N. S. Azhagarasan M.D.S.,
Principal,
Ragas Dental College & Hospital,
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI-600 119.

THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY

CHENNAI

PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **"IDENTIFICATION OF SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING TECHNOLOGY"** of the candidate **DR. ANISHA DEBORAH JEYAKUMAR** for the award of **MASTER OF DENTAL SURGERY** in the branch of **PERIODONTOLOGY**.

I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **3 percentage** of plagiarism in the dissertation as per the report generated.

Date: 25-01-2018
Place: Chennai.

Guide & Supervisor sign with Seal.

Dr. Ramya Arun, M.D.S.,
Reader,
Department of Periodontology,
Ragas Dental College & Hospital
Chennai.

Department of Periodontics,
Ragas Dental College & Hospital,
2/102, ECR, Chennai-119

Acknowledgement

ACKNOWLEDGEMENT

First of all I would like to thank **LORD JESUS CHRIST**, for bestowing His everlasting Grace and Mercy upon me, without which pursuing this degree would have been an impossible uphill task in my life.

I take this opportunity to thank **Dr. N.S. Azhagarasan, MDS**, Principal, Ragas Dental College and Hospital for his support and guidance during my postgraduate course at Ragas Dental College and Hospital.

I express my sincere thanks to my respected and beloved Professor **Dr. K.V. Arun**, **MDS**, Professor and Head of the Department of Periodontics, Ragas Dental College Chennai, for his valuable advice, guidance and encouragement during my postgraduate course. I am deeply grateful to him for his patience and guidance during the study process.

I express my sincere gratitude to my beloved guide, **Dr. Ramya Arun , MDS,** Reader, Department of Periodontics, Ragas Dental College and Hospital for guiding me through the strenuous path of dissertation and providing me with her valuable advice, guidance, support and encouragement during the entire period of my postgraduation.

I also extend my gratitude to **Dr. G. Sivaram, MDS,** Professor, **Dr. B. Shiva Kumar, MDS,** Professor, for their continuous guidance and constant encouragement throughout my study period.

I would like to express my profound sense of gratitude to **Dr. Swarna Alamelu, MDS,** Reader and **Dr. Archana Meenakshi, MDS,** for their constant support and encouragement throughout my tenure.

I would like to thank **Dr. Radhabharathi, MDS,** Senior Lecturer, **Dr. Deepavalli, MDS,** Senior Lecturer and **Dr. A.R. Akbar, MDS,** Senior Lecturer for their continuous support and guidance. I would also like to thank **Dr. R.S. Pavithra, MDS,** Senior Lecturer, **Dr. J. Velkumar, MDS,** Senior Lecturer and **Dr. M. Divya, MDS,** Senior Lecturer for their constant support.

I remain ever grateful to my batch mates **Dr.Arvinth, Dr.Gayathri, Dr. Latha, Dr. Manimalla** and **Dr. Sakthi Ganesh,** for their constant support and encouragement. I thank all my seniors, especially **Dr.Guhanathan** and **Dr.Kalaivani**, and my juniors, especially **Dr.Cynthia** for their help, support and encouragement.

I extend my gratitude to **Mrs. Parvathi, Mrs. Rosamma, Mr. Chellapan, Mrs. Mala** and **Ms. Sheela** for their timely help during the tenure.

I would like to thank **Dr. Aarthy M.D.S., Dr. Anusha M.D.S.,** and **Mr. Mythreya,** for helping me with the statistical part of this dissertation.

I would like to thank my parents **Cdr. Douglas Jeyakumar** and **Dr. Shobhana Jeyakumar** for their love, understanding, support and

encouragement throughout these years. I would also like to thank my in-laws, **Dr. Asirvatham Jayaseelan** and **Mrs. Malathi Jayaseelan** for supporting me emotionally throughout my postgraduation.

I would like to express my heartfelt love and gratitude to my husband **Dr. Jason Paul Danny,** for his belief in me and constant support and encouragement throughout my course. Without his support this course and this study would not have been possible.

LIST OF ABRREVIATIONS

ABBREVIATION	EXPANSION
PSD	• Polymicrobial Synergy and Dysbiosis
DNA	• Deoxyribonucleic acid
rRNA	• Ribosomal Ribonucleic acid
HOMIM	• Human Oral Microbiome Identification Microarray
NGS	• Next Generation Sequencing
MSR	• MiSeq Reporter software
HOMD	• Human Oral Microbiome Database
OTU	• Operational Taxonomic Unit
PCR	• Polymerase Chain Reaction
SoLiD	• Supported Oligonucleotide Ligation and Detection
BLAST	• Basic Local Alignment Search Tool
HOT	• Human Oral Taxon number

CONTENTS

LIST OF TABLES

LIST OF GRAPHS

LIST OF FIGURES

Introduction

INTRODUCTION

Human body is estimated to be composed of more than 10^{14} cells of which only 10% are mammalian.[98] Microorganisms contribute to majority of cells found in our body, forming resident microflora, surviving in symbiotic relationship with the host and thus maintaining homeostasis.

Oral microbiome exhibits the largest core of commonly related microbes among unrelated subjects when compared to gut or skin microflora.[18,59,136] The relationship between periodontal microflora and host is known to be benign, but changes in subgingival microbiome and bacterial community structures can contribute to pathogenesis of periodontal disease.[49]

Periodontal diseases are a heterogeneous group of chronic conditions affecting tooth supporting structures.[126] It is described as a dysbiotic disease characterized as being polymicrobial and multifactorial in nature. Dysbiosis is a symbiotic relationship which has gone astray caused by a decrease in number of beneficial symbionts and/or an increase in number of pathobionts.[12]

Research over the past decade has led to recognition of microbes residing in various oral ecological niches as a part of dental plaque. Plaque biofilm is a highly organized accumulation of microbial communities adhering to favourable environmental surfaces, which functions to maximize energy, spatial arrangements, communication, and continuity of bacterial communities. The origin, development and structural adaptation of dental plaque is governed by a dynamic, ever-changing equilibrium between oral

microbiota and multiple factors that differentially promote or inhibit survival of its microbial constituents.[62]

According to previous studies by **Socransky et al**,[105] the key bacterial species which play a role in the disease process have been segregated into microbial complexes based on their correlation with clinical parameters and severity of periodontal disease. Over the past 50 years, understanding and characterization of dental plaque has undergone significant evolution from nonspecific plaque hypothesis[13,76] and specific plaque hypothesis[66] to Ecological Plaque Hypothesis.[72] Currently, the pathogenesis of periodontal diseases is explained by "Polymicrobial Synergy and Dysbiosis (PSD) Model" proposed by **Hajishengallis et al**.[35] This model states that dysbiotic environment and polymicrobial synergy are the key events that lead to development of periodontitis rather than individual bacterial species. Thus biofilm community as a whole was thought to be either health-associated or disease-associated.

Abnormal pocket depth and pocket-free gingival recession have been recognized as two separate periodontal phenotypes at least since the 18th century.[43] Gingival recession refers to exposure of root surfaces caused by apical displacement of gingival margin beyond cemento-enamel junction.[125] The three primary etiologic agents of gingival recession are plaque-induced inflammation, mechanical abrasion/removal due to faulty tooth brushing, and developmental/mucogingival deformities. In the Indian subcontinent an important reason for gingival recession is accumulation of local factors due to

inadequate oral hygiene practices. Several studies on subgingival microbiome have documented data regarding periodontal pocket,[54,79,87,105] but there is paucity in literature regarding subgingival microbiome in gingival recession sites.

Traditional culturing methods for bacterial identification are often insufficient for biofilm analysis due to unknown culture characteristics of several bacterial species; hence a vast majority of oral bacteria evade standard microbiological detection methods. This has led to development of alternative methods to assess dental biofilms based on DNA analysis or other molecular techniques.[41] Studies of subgingival microbial community identified a link between taxonomic composition and disease pathogenesis. Current trend in sequencing of microbiome is based on Next-Generation Sequencing (NGS), that uses parallel sequencing of multiple small fragments of DNA to determine genetic sequences.[93] The speed of sequencing and amounts of DNA sequence data generated with NGS, which is considered a "high-throughput technology", are exponentially greater and are produced at significantly reduced costs.[7] NGS technology was utilized earlier in our department (unpublished data) to identify and characterize subgingival microbiome from periodontal pockets.

Subgingival community has been extensively studied earlier to characterize their taxonomic composition.[79,87,105] The difference in functional profile of subgingival microbiome in periodontal health and disease is

perplexing and identifying these changes offer a promising potential for research in pathogenesis of periodontal disease.[102]

For a better understanding of the etiology and polymicrobial mechanisms of localized chronic periodontitis presenting as gingival recession due to plaque-induced bacterial inflammation, the current study was undertaken to identify, characterize and analyse species of subgingival microbiome and characterize novel micro-organisms among periodontal health and disease using NGS technology.

Aim and Objectives

AIM AND OBJECTIVES

Aim:

To identify and characterize subgingival microbiome using Next Generation Sequencing Technology in periodontal health and chronic periodontitis presenting as gingival recession.

Objectives:

1. To identify and characterize the subgingival microbiome in periodontal health and in gingival recession sites using Next Generation Sequencing Technology.
2. To compare and analyze subgingival microbiome in sites of gingival recession with those of periodontally healthy controls.
3. To study species diversity in subgingival microbiome, and characterize novel micro-organisms in gingival recession sites for a better understanding of the role in pathogenesis.

Review of Literature

REVIEW OF LITERATURE

Mouth as a microbial habitat:

The human body is estimated to be composed of more than 10^{14} cells, of which only 10% are mammalian.[98] The majority of these cells are the microorganisms that make up the resident microflora found on all environmentally exposed surfaces of the body, and this human "microbiome" is reported to have a metabolic capacity equivalent to that of the human liver.[71] It is estimated that between 300 and 400 different species are capable of colonizing the mouth, and any individual may typically harbor 150 to 200 different species. Counts in subgingival sites range from about $l0^3$ in healthy, shallow sulci to more than 10^8 in deep periodontal pockets.[106] The oral microflora is distinctive because of the characteristic biological and physical properties of each site despite potential movement of microorganisms between sites. Bacteria may attach to tooth itself, to epithelial surfaces of gingiva or periodontal pocket, to underlying connective tissues, if exposed, and to other bacteria attached to these surfaces. These observations illustrate a key concept that properties of the habitat are selective and dictate which organisms are able to colonize, grow and be minor or major members of the community.[71]

HUMAN ORAL MICROBIOME:

Concept of the human oral microbiome

The microorganisms found in human oral cavity have been referred to as oral microflora, oral microbiota, or more recently as oral microbiome. The

term microbiome was coined by Joshua Lederberg "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease". **Turnbaugh et al (2007)**[118] described human microbiome to be classified into a core microbiome and a variable microbiome. Core microbiome comprises of the predominant species that exist under healthy conditions at different sites of the body, and it is shared by all individuals.[109,118,132] Variable microbiome is one that has evolved in response to unique lifestyle, phenotypic and genotypic determinants; it is exclusive for an individual. Even though individuals share microbiota at similar sites of the body, varying differences are observed at species and strain level of the microbiome which may be as unique as a fingerprint to an individual.[20]

The oral cavity is highly populated with numerous polymicrobial communities, each occupying highly specific niches that differ in both anatomic location and nutrient availability. Oral host colonization is a reflection of bacterial proficiency to adapt to a variety of different niches through high rates of genetic recombination.[94] Oral microbiota needs to cope with daily physical and chemical perturbations from intake of food and personal hygiene measures, and these include fluctuations in temperature, pH, antimicrobial and dietary components, and mechanical sheer forces from brushing and mastication.[133] Recent data from the Human Microbiome Project (HMP) revealed that oral microbiome has the largest core of commonly shared

microbes among unrelated individuals compared to other habitats such as gut or skin.[18,59,136]

Dysbiosis is defined as change/perturbations in the structure and composition of resident commensal bacterial communities relative to the community found in healthy individuals.[89] Dysbiosis, as the term implies, is a symbiotic relationship which has gone astray caused by a decrease in the number of beneficial symbionts and/or an increase in the number of pathobionts.[12] Biological properties that help to maintain stability in the microbiome are important for sustaining symbiotic environment and for prevention of dysbiotic state.

Constitution, acquisition and maintenance of the normal oral microbiome

Human oral cavity is colonized by a wide range of microorganisms. Besides bacteria and fungi, Archaea, viruses and protozoa form a part of normal microbiome.[122] The microbiome has been studied in great detail and phylogenetic information of oral bacteria is gathered in databases dedicated to oral cavity.[86] The HMP assessed microbiome composition of nine intraoral sites (buccal mucosa, hard palate, keratinized gingiva, palatine tonsils, saliva, subgingival and supragingival plaque, throat and tongue dorsum) from about 200 subjects and found 185 to 355 genera, belonging to 13 to 19 bacterial phyla.[136] The microbiome has evolved through hundreds of thousands of years of co-habiting into a microbe-human symbiosis with mutual benefits. **Costello et al**[18] reported that oral microbiome in newborns was shown to seed the gut microbiome that first resembles that of the oral cavity and diverges in

2 weeks' time to gut-specific communities. Based on current knowledge it is apparent that acquisition of such normal, beneficiary microbiome by newborns is an essential process. Infants are colonized rapidly after birth by bacteria present in their direct environment, through bacterial transfer from their mother but also from other sources.

Zaura et al[133] has suggested that development of fetal tolerance towards microbiome of the mother during pregnancy is a major factor for successful acquisition of a normal microbiome. Although the first encounter of a newborn with microbiota is considered to be postnatal, several studies have shown clinical evidence for microbial presence in placenta, umbilical cord blood, amniotic fluid, and meconium in full-term pregnancies without overt infection.[1,10,45,111] Vertical transmission from mother to child starts at birth. The delivery mode (vaginal or Caesarian), to a large extent, determines which microorganisms—vagina or skin-derived— will be encountered first by the new born. This affects diversity of oral microbiome: vaginally born infants showed higher taxonomic diversity at 3 months of age.[61] **Li et al**[59] reported that birth mode may have a lasting impact as infants born with Caesarian section acquired *Streptococcus mutans* almost 1 year earlier (at 17.1 months of age) than vaginally born infants (28.8 months). The method of feeding (breast-feeding or infant formula) affects the infant's microbiome as well. Breast-fed infants of 3 months of age carried oral lactobacilli with antimicrobial properties not found in formula-fed infants.[133]

Once established, the microbiome should be sustained. This is reported to be achieved through host-derived and microbe-derived microbiome maintenance factors. The interactions between the microbiome and the host are bidirectional, and the factors that determine the continued communication routes include innate and adaptive immune responses, host pattern recognition receptors (Toll-Like Receptors play a key role), chemosensory activation mechanisms, crevicular and salivary glycoproteins.

Co-evolution of the microbiome with host has resulted in host-associated microbial communities that are equipped with mechanisms that allow them to prevent colonization and establishment of foreign microbes, so called "colonization resistance".[40] Integrity of the microbial community is maintained by specific inter-microbial adhesion, cell signaling through cell-to-cell contact, metabolic interactions and quorum sensing. Besides bacterial inter-species communication, inter- kingdom communication also plays a role in oral microbial ecosystem.[44,80]

PERIODONTAL DISEASE- ETIOLOGY AND PATHOGENESIS

Periodontal diseases are a heterogeneous group of chronic conditions that reflect a cellular inflammatory response of supporting periodontal tissues of the teeth against bacterial challenges.[90] It is a dysbiotic disease characterized as being polymicrobial and multifactorial in nature exhibiting a shift from predominantly gram-positive bacteria found in healthy sites to mostly gram-negative bacteria found in clinically diseased sites. The initiation and progression of the inflammatory and destructive periodontal lesion is

related to the lack or minimal proportions of beneficial microorganisms in a susceptible host.[90] The end outcome of untreated periodontal disease is loss of attachment apparatus and subsequent loss of teeth often leaving patients unable to eat and function properly.

Löe et al (1978)[65] demonstrated the natural progression of periodontal disease through a series of studies over several years following a population of Sri Lankan tea workers which represented a relatively uniform population that had little to no dental care and also had extremely poor oral hygiene. Several lines of evidence indicate that bacteria are necessary for the development of inflammation in the periodontal tissues. In a study by **Mitchell and Johnson**[77] bacteria were implicated in periodontal disease with the observation that administration of penicillin inhibited periodontitis in laboratory animals, and **Keyes and Jordan**[48] demonstrated the infectious nature of periodontitis by its transmissibility in animal models.

The current concept concerning the etiology of periodontal disease considers three groups of factors which determine whether active periodontal disease will occur: a susceptible host, presence of pathogenic species, and absence of so-called "beneficial bacteria".[47] It has been demonstrated that initiation and progression of inflammatory and destructive periodontal lesions is related not only to presence of bacterial strains pathogenic for the periodontium, but also to lack or minimal proportions of beneficial microorganisms in a susceptible host. The unequivocal role of dental bacterial plaque in development of gingivitis and periodontitis was established almost

40 years ago. Gingivitis is a reversible inflammatory reaction of marginal gingiva to plaque accumulation, whereas periodontitis is a destructive, nonreversible condition resulting in loss of tooth connective-tissue attachment to bone, which ultimately leads to loss of the involved teeth. Existing evidence indicates that gingivitis precedes onset of periodontitis; however, not all gingivitis cases develop into periodontitis. The reason for this is that accumulation of plaque bacteria is necessary but not sufficient by itself for development of periodontitis: a susceptible host is necessary.[114]

GINGIVAL RECESSION

According to **Wensstrom JL (1996)**[125] gingival recession refers to exposure of root surface caused by apical displacement of gingival margin beyond cementoenamel junction. Gingival recession, either localized or generalized, is one of the clinical features of periodontal disease and is frequently associated with clinical problems such as root surface hypersensitivity, root caries, cervical root abrasions, erosions, plaque retention and aesthetic dissatisfaction.[15]

Abnormal pocket depth and pocket-free gingival recession have been recognized as two separate periodontal phenotypes at least since the 18th century.[43] The distinction as two different phenotypes was summarized by Page & Sturdivant[85] as:

1. Periodontal atrophy, where the gingiva retain a very healthy aspect and are quite free of pain and inflammation, and yet will gradually recede

2. Destructive periodontal disease with presence of deepened periodontal pockets and underlying bone loss

Etiology of gingival recession:

The primary causes of gingival recession are plaque-induced inflammation and mechanical (physical) abrasion/removal. Occasional causes of recession include thermal and chemical injury. Three major factors are associated with increased susceptibility to gingival recession: (i) thin gingival tissue; (ii)mucogingival conditions; and/or (iii) a positive history of progressive gingival recession and/or inflammatory periodontal diseases in teeth presenting with either or both of the first two factors.[74] Mucogingival conditions are defined as deviations from the normal anatomic relationship between the gingival margin and the mucogingival junction.

The etiology is multifactorial and includes excessive or inadequate teeth brushing, destructive periodontal disease, tooth malposition, alveolar bone dehiscence, high muscle attachment, aberrant frenal pull, occlusal trauma, iatrogenic factors (such as orthodontic, or prosthetic treatment) and smoking.[55]

Pathogenesis and severity of recession:

The mechanism of gingival recession due to localized inflammatory processes in connective tissues with the accumulation of mononuclear cells was described by **Baker and Seymour (1976)**.[9] In the initial stage there is normal or subclinical inflammation, following this inflammation appears

clinically and histologically where there is proliferation of epithelial rete pegs. Stage three shows increased epithelial proliferation resulting in loss of connective tissue core and finally there is merging of oral and sulcular epithelium resulting in separation and recession of the gingival tissues due to loss of nutritional supply.

Waerhaug J (1952)[123] proposed that the distance between the periphery of plaque on the tooth surface and the labial, apical extension of the inflammatory infiltrate hardly ever exceeds 1-2 mm. Thus if the free gingiva is voluminous the infiltrate will occupy only a small portion of the connective tissue; however if it is thin the entire connective tissue portion may be involved, consequently there is proliferation of epithelial cells from the oral and dentogingival epithelium, the zone of connective tissue decreases and finally recession ensues.

Sarfati et al (2010)[100] reported that gingival bleeding was significantly associated with gingival recession severity and concluded that inflammatory reaction to dental biofilms is the predominant biologic feature shared by gingival recession and periodontitis. In his study, he found that gingival bleeding was significantly associated with severity of gingival recessions (P value = 0.010) and not with extent of gingival recessions, which suggested that extent of recession is related to plaque accumulation and that host response, i.e., inflammation conditions, is a key factor for recession severity.

Similarly, number of missing teeth was associated with severity of gingival recession and not with extent of gingival recession. When there is

more tooth loss, it has been stressed that there is more periodontitis, attachment loss, and consequently, gingival recession. This finding illustrates that periodontitis is a site-specific disease, which means that number of missing teeth does not depend on extent of the disease but rather on defect severity. Thus the number of missing teeth could be a better surrogate criterion of recession severity than extent of recession. **Van der Velden et al**[119] showed an association between gingival recession and periodontitis severity in a Java population. This outcome, together with results from the study by Sarfati in 2010, may indicate a bi-directional association between gingival recession severity and periodontitis severity, rather than a causal relationship. It may be assumed that inflammatory reaction to dental biofilm is the predominant biologic feature shared by gingival recessions and periodontitis.

Keratinized tissue width as a parameter for gingival recession:

Lang and Loe (1972)[58] demonstrated that although tooth surfaces may be kept free of clinically detectable plaque, areas with less than 2 mm of keratinized gingiva tend to remain inflamed. However, clinicians like **Dorfman HS**[24]**, Freedman AL et al**[29]**, Kisch et al**[50] and **Miyasato**[78] have evaluated sites with less than 2 mm of keratinized tissue and concluded that these sites do not necessarily develop gingival recession solely as a result of a narrow width/band of keratinized tissue. **Serino et al**[101] reported that teeth with a positive history of progressive gingival recession have increased susceptibility to additional apical displacement of soft-tissue margin.

In **1999**, **Albandar and Kingman**[5] suggested that gingival recession occurs primarily as a consequence of periodontal diseases and aggressive use of mechanical oral hygiene measures. A positive history of inflammatory periodontal disease (e.g. plaque-induced gingivitis and localized chronic periodontitis) can be considered an important factor associated with gingival recession, especially for teeth with thin gingival tissues and/or mucogingival conditions.

ETIOLOGIC ROLE OF PLAQUE IN PERIODONTITIS

Plaque biofilm as an etiological agent for periodontitis

The microbial etiology of periodontitis has been extensively researched for the past few decades, and it is now well known that periodontitis is not associated with a single micro-organism but is a consortium of bacteria participating in initiation and progression of disease process.[96] The most significant consequence of biofilm formation on tooth surfaces is continuous release of bacterial toxins into the local microenvironment. The composition of subgingival microbial flora and level of pathogenic species differ from subject to subject as well as from site to site. **Marsh (2011)**[71] described dental plaque as the microbial community that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. Dental plaque forms via an ordered sequence of events resulting in a structurally and functionally organized species-rich microbial biofilm.

Changing views on etiologic role of dental plaque

According to an experimental gingivitis study by **Loe (1965)**[65], etiologic role of plaque in periodontal disease and its involvement in initiation and progression of periodontal diseases was firmly established. It has been generally accepted that periodontal diseases appear to be initiated by a relatively limited number of periodontal pathogens in the complex dental biofilm, and they represent a small part of approximately 600 bacterial species that have been found to colonize dental surfaces over and below the gingival margin and oral mucous membranes.[90] Clinical and experimental evidences in last three decades confirm that certain bacterial strains in the periodontal environment can induce gingival tissue inflammation and bone destruction, and were termed as periodontal pathogens.[83] Only a small percentage of dental biofilm bacteria are defined as pathogenic for periodontal tissues and these bacteria, even when present in very small quantities, possess the ability to damage periodontal structures.

According to the criteria proposed by **Socransky and Haffajee**[108], a microorganism must meet certain conditions to be considered a potential periodontal pathogen: association with disease by means of increased number in diseased patients and sites; reduction or elimination after treatment; capable of provoking destructive host responses; capacity to cause disease in experimental animal models; ability to produce virulence factors known to cause periodontal destruction. On the basis of the proposed criteria by Sockransky's modifications of Koch's postulates, several bacteria have been

examined and shown to be periodontopathogens and disease initiators of periodontitis.[83] Over the past 50 years, ideas about changes in plaque relating to shift from oral health to disease, and understanding and characterization of dental plaque have undergone significant evolution.

Non-specific plaque hypothesis

Based on work of researchers **Black**[13] and **Miller**[76] the non-specific plaque hypothesis was proposed which stated that *periodontal disease is due to bacterial accumulation, irrespective of its composition.* This implies that no one specific bacterial species is any more significant than another in its ability to cause periodontal disease.[39] Increase in quantity of plaque, as opposed to specific pathogenic microorganisms (quality) found in plaque, were viewed as being primarily responsible for inducing disease and disease progression.[33]

Although the amount of plaque present may correlate well with disease severity in cross-sectional studies, it correlates poorly in longitudinal studies. Non-specific plaque hypothesis is valid for development of gingivitis but not for periodontitis, which is a multifactorial disease.[84] This concept failed to explain why all gingivitis do not progress to periodontitis, why some individuals with increased plaque showed little overt periodontitis and why some individuals with very little plaque manifested with aggressive and advanced forms of periodontitis.[108] This hypothesis did not consider variations in dental biofilm that may affect its pathogenicity or, most importantly, host determinants.

Specific plaque hypothesis

Specific plaque hypothesis proposed by **Walter J. Loesche**[66] stated that *periodontal disease is the result of an infection with a single specific pathogen.* Studies on microbial etiology of various forms of periodontitis support this hypothesis, which proposes that only certain microorganisms within the plaque complex are pathogenic. **Newman MG**[82] and **Slots**[104] identified *Aggregatibacter actinomycetemcomitans* as a specific pathogen in localized aggressive periodontitis. Despite the presence of hundreds of species of microorganisms in periodontal pockets, fewer than 20 are routinely found in increased proportions at periodontally diseased sites. These specific virulent bacterial species activate the host's immune and inflammatory responses that then cause bone and soft tissue destruction. This hypothesis failed to explain why putative periodontal pathogens like *Porphyromonas gingivalis*, *Tannerella forsythia* are frequently found in healthy periodontal sites.

Based on this concept, it was recognized that early plaque consists predominantly of gram-positive organisms and if left undisturbed it undergoes a process of maturation resulting in a more complex and predominantly gram-negative flora. **Sockransky et al**[105] assigned organisms of subgingival microbiota into groups, or complexes, based on their association with health and various disease severities.[33] The yellow, green and purple complexes were early colonizers that favour colonization of orange and red complexes. Red complex bacteria included *Bacteroides forsythensis* (now *Tannerella*

forsythia), *Porphyromonas gingivalis* and *Treponema denticola* and they were significantly associated with periodontitis. (Figure 1)

Ecological plaque hypothesis

Ecological plaque hypothesis, proposed by **Philip D. Marsh (1994)[72],** described and explained the dynamic relationship between resident microflora and host in health and disease in ecological terms. The theory underpinning this hypothesis in the context of periodontal disease is that changes in the environment increase competitiveness of putative pathogens at the expense of species associated with oral health and upregulate the expression of virulence factors. **Marsh et al[71]** reported a clear link between local environmental conditions and activity and composition of the biofilm community; any change to the environment induces a response in the microflora and vice versa.

Keystone Pathogen Hypothesis

Socransky et al[105] reported that when disease develops and advanced stages are reached, the keystone pathogens are detected in higher numbers. Keystone Pathogen Hypothesis, proposed by **Hajishengallis et al (2012)[35],** indicates that certain low-abundance microbial pathogens can cause inflammatory disease by increasing the quantity of normal microbiota and by changing its composition.

Polymicrobial Synergy and Dysbiosis Model (PSD):

PSD model of pathogenesis described by **Hajishengallis et al[35]** states that periodontitis is initiated by a broadly based dysbiotic, synergistic microbiota as against the traditional view that it is caused by a single or

several periopathogens like red complex bacteria. This dysbiotic, synergistic microbiota alters host-microbe homeostasis and facilitate its transition to a chronic inflammatory state. **Lamont and Hajishengalis**[57] observed that the whole microbial community drives disease progression, representing the interplay between subgingival community of microorganisms and local immune responses which ultimately drives bone and connective tissue attachment loss.

SUBGINGIVAL MICROBIOME

Members of the human oral microbiome were among the first bacteria ever to be observed. In 1683, Antonie van Leeuwenhoek used his microscope to observe a large number of what he named "animalcules" in scrapings taken from his teeth. Over 200 years later, the seminal work of Koch, Pasteur and their contemporaries identified the animalcules as microorganisms and the first isolates of cultivable members of the oral microbiome were studied in the laboratory.

The subgingival microbiome is the community of microorganisms inhabiting the subgingival environment. **Haffajee and Socransky**[34] and **Zambon**[131] have extensively studied the microbial composition of subgingival plaque at periodontally diseased sites. In a landmark study by **Socransky and Haffajee**[105], they attempted to define bacterial communities existing as different complexes in subgingival plaque by studying 13,261 plaque samples from 185 subjects using whole genomic DNA probes and checkerboard DNA-DNA hybridization. They defined 5 major bacterial complexes identified by

different clustering and ordination techniques. The complex most significantly associated with periodontitis and to clinical measures like probing depth and bleeding on probing was the red complex, comprising of *Tanerella forsythia, Porphyromonas gingivalis and Treponema denticola.*[105] A series of culture studies by **Moore and Moore**[79] involving analysis of subgingival plaque taken from subjects with different forms of periodontal disease and health reported a shift in the subgingival microbiota as the periodontium progressed from health through gingivitis to periodontitis. **Liu et al**[63] and **Chen et al**[16] investigated bacterial diversity between periodontal health and disease status using 16S rRNA amplicon sequencing and showed that there is a shift in the composition of the oral microbiota between healthy and diseased samples.

Kumar et al (2003)[54] investigated subgingival microbiome based on 16S rDNA cloning and sequencing and showed that 40% of bacterial species present were either novel species or phylotypes. **Paster BJ and Dewhirst (2006)**[87] developed a molecular technique to detect oral biofilms using a 16S rRNA-based microarray technology called Human Oral Microbiome Identification Microarray (HOMIM). This system provides information on the 9 most commonly found oral bacterial flora namely *Bacteroidetes, Firmicutes, Proteobacteria, Synergistetes, Fusobacteria, Spirochaetes, Actinobacteria, SR-1* and *TM-7.*

DETECTION AND ENNUMERATION OF BACTERIAL SPECIES FROM PERIODONTAL SAMPLES:

Microscopic studies

In 1683 Antonio van Leeuwenhoek studied scrapings from human teeth using microscope and described 5 types of animalcules. **Coyler** had proposed use of dark field microscopy for evaluation of pocket microorganisms.[91] **Theodore Rosebury** conducted a series of experiments to isolate bacteria of etiological importance in periodontal disease. **Keyes (1965)**[48] proposed use of phase contrast microscopy in periodontal diagnosis to identify bacterial morphotypes.

Microscope techniques were reasonably rapid, but limited in the precision of identification of individual bacterial species. The strength of microscopy techniques is delineation of spatial arrangements of organisms; whereas its weakness from an ecologic perspective is that they are slow, labor intensive and precise speciation using immunologic or hybridization techniques can only be performed for a very limited number of species in any given sample.[107]

Culture based methods

Cultivation of organisms and identification of species by their phenotypic traits was one of the major techniques used by researchers to identify plaque bacteria. The classic studies of **Moore & Moore**[79] in which they examined composition of subgingival plaque samples in periodontal health and different periodontal disease states employed cultural techniques to

examine over 17,000 isolates from over 600 periodontal sites. Although this was a massive amount of work, it was considered a limited number of samples by current standards. The major strength of culture is that majority of bacterial species sampled can be grown and identified in lab conditions. The main drawback of culture method is its narrow spectrum, and it is regarded as a time-consuming, labor-intensive, and expensive undertaking because only few plaque samples in small numbers of subjects can be examined. Studies by **Kolenbrander (2000)[51], Vartoukian et al (2007)[120]** and **Siqueria et al (2010)[103]** have estimated that 50% to 60% of distinct bacterial phyla in oral cavity still have no cultivable representatives. However, cell culture is still essential to assess bacterial sensitivity to antibiotics and for verifying presence of known species.

Immunologic and enzymatic assays

Immunofluorescence techniques and enzyme-linked immunosorbent assay (ELISA) techniques are antibody-based methods used to enumerate specific species of microorganisms without their cultivation; it is dependent on specificity of developed antibodies to specific taxa. These techniques have the advantage that samples do not have to be cultured for enumeration; they are rapid and less expensive than culture. However, they are limited to species for which reagents have been developed; it is difficult to use these techniques to evaluate species in large numbers of plaque samples and it is time-consuming to develop and validate specific antisera to new species.[107]

DNA – DNA hybridization or checkerboard

DNA-DNA hybridization is a molecular approach used to detect bacteria based on hybridization of target species to labeled genomic DNA that has been attached to nylon membranes. Studies by **LoescheWJ et al[66], Haffajee[34], Ximenez-Fyvie LA[129], Feres M et al[27]** and **Socransky[107]** have reported on levels of limited number of species with this method in adult periodontitis, periodontal health, refractory periodontitis and response to therapy. This method provides a major benefit for studies of oral microbial ecology due to advantages like detection of multiple species from each sample simultaneously, and study of large sample size for large numbers of species. Checkerboard technique is rapid, sensitive, and relatively inexpensive but is also dependent on culture technique to cultivate the target species for creating genomic probes. Like antibody-based assays, cross reactivity can be verified only with cultivated species hence specificity of the probe is an unknown variable.

Polymerase chain reaction

Kary Mullis first developed polymerase chain reaction (PCR) technique to amplify specific genes or parts of genes which are then used to identify bacterial species from which they originated.[53] In a study by **Kumar PS et al (2005)[54]** species-specific PCR primers were designed and used in individual PCR reactions to detect prevalence of target species in plaque samples of healthy and diseased subjects. These studies confirmed that several species, including uncultivated ones, were associated with oral health or

periodontitis. Given the appropriate primers, this method is rapid, simple, can detect small numbers of cells of a given species, and indicates the presence or absence of a species in the sample. It has certain disadvantages of not providing quantitative data, may not be cost effective for large sample sizes, and for applications where relative levels of species are important, PCR may not be ideal.[107]

DNA Probes

Oligonucleotide probes are short probes designed to identify unique regions of DNA within cells of a given bacterial species. These probes are highly specific and likelihood of cross-reactions with other species is very low. Because they target a limited segment of DNA of an organism, oligonucleotide probes tend to be less sensitive for detection of low numbers of bacteria than whole genomic probes.[107]

Whole genomic DNA probes are constructed using the entire genome of a bacterial species as the target and thus can be quite sensitive. The use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. The technique can detect only species for which DNA probes have been prepared, thus novel pathogens or environmentally important species that might be detected in culture or by other molecular techniques may not be identified.[114]

OPEN ENDED APPROACHES- 16S rRNA sequencing analysis:

Open ended approaches allow identification of even uncultivated and previously unknown species. According to **Spratt (2004)**[110] these approaches are based on 16 S rRNA sequencing, amplification and analysis of the 16S rRNA genes in a microbiome sample. 16S rRNA has proven to be the most useful phylogenetic marker to identify bacteria and to determine their evolutionary relationships. Ribosomal RNA gene is essential for life and present in all prokaryotes. It contains nucleic acid sequences with highly conserved and variable regions; conserved regions are used to design universal PCR primers capable of recognizing segments of 16S rRNA gene sequence of all bacterial species and hypervariable regions are used as signatures to discriminate one species from another. 16S rRNA gene is large enough (about 1500 bases) to provide sufficient sequence variability among bacteria, thereby making comparisons possible at different taxonomic levels.

DNA and protein sequencing started in the 1970s when the virus Lambda (50,000 nucleotides) was sequenced by **Sanger et al.**[97] **Frederick Sanger** and colleagues described the use of chain-terminating dideoxynucleotide analogs that caused base-specific termination of primed DNA synthesis and this came to be popularly known as Sanger sequencing method.[107] This method of sequencing was considered the gold standard, and over the years, whole genome sequencing of many bacteria has been carried out using this method. Sanger technology was used in the sequencing of the first human genome, which was completed in 2003 through the ***Human***

Genome Project, a 13-year effort with an estimated cost of 2.7 billion dollars.[121]

Over the past decade, next generation sequencing technologies have emerged, which are high throughput and able to generate three to four orders of magnitude more sequences and are also relatively less expensive.[23]

NEXT GENERATION SEQUENCING TECHNOLOGY (NGS):

Next generation sequencing methods employ a wide spectrum of technologies such as sequencing by synthesis, sequencing by ligation, single molecule DNA sequencing and colony sequencing. NGS is performed by repeated cycles of polymerase-mediated nucleotide extensions or by machinery automated cyclical ligation of oligonucleotides.[69,121]

Fundamentals of NGS platforms:

NGS platforms share a common technological feature—massive parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell. This design is a paradigm shift from that of Sanger sequencing, which is based on electrophoretic separation of chain-termination products produced in individual sequencing reactions. In NGS, sequencing is performed by repeated cycles of polymerase-mediated nucleotide extensions or, in one format, by iterative cycles of oligonucleotide ligation.[121] As a massively parallel process, NGS generates hundreds of megabases to gigabases of nucleotide sequence output in a single instrument run, depending on the platform. The 2 basic procedures are ligation of DNA

fragments with oligonucleotide adaptors and fragment immobilization to a solid surface, such as a bead.

The three commonly used platforms for massive parallel DNA sequencing at present are the Roche/454 FLX (Life Sciences, Branford, CT, **Margulies et al., 2005**)[70], the Illumina/ Solexa Genome Analyzer (Illumina, San Diego, CA, Bentley DR, 2006, **Korbel et al., 2007**)[52] and the Applied Biosystems / SOLiD (Life Technologies, Carlsbad, CA).[69,121] The most recent powerful NGS platforms have significant reductions in the run time and remarkable data output, they include HiSeq and the Ion Torrent Personal Genome Machine (PGM).[95]

ROCHE 454 LIFE SCIENCES SYSTEM

The 454 technology is derived from technological convergence of pyrosequencing and emulsion PCR. In 2000, Jonathan Rothberg founded 454 Life Sciences, which developed the first commercially available NGS platform, GS 20, launched in 2005.[121] One of the major drawbacks of this system is that sometimes more than one nucleotide is incorporated in DNA template during a cycle, making it difficult to resolve homopolymeric stretches of sequence (e.g. CCCCC or AAAAA). (Figure 2)

ILLUMINA/SOLEXA GENOME ANALYZER

In 1997, British chemists Shankar Balasubramanian and David Klenerman conceptualized an approach for sequencing single DNA molecules attached to microspheres and founded Solexa in 1998. The Solexa Genome Analyzer, the first "short read" sequencing platform, was commercially

launched and acquired by Illumina in 2006. Genome Analyzer uses a flow cell with bound oligonucleotide anchors wherein template DNA is fragmented into several hundred base pairs and end-repaired (Figure 3).[121]

The newest platform, the Genome Analyzer II, has optical modifications enabling analysis of higher cluster densities. Illumina and other NGS technologies have devised strategies to sequence both ends of template molecules. Such "paired-end" sequencing provides positional information that facilitates alignment and assembly, especially for short reads.

The advantage of Solexa system is that it can generate 1.5 GB of sequence per run with read lengths that range from 35 to 100 bases and each run requires 3–5 days to complete.[95] A technical concern of Illumina sequencing is that base-call accuracy decreases with increasing read length primarily due to "dephasing noise" which occurs when a complementary nucleotide is not incorporated or when fluorophore is not properly cleaved at the end of cycle, thus blocking incorporation of next nucleotide base.[121] As a consequence, the sequence is out-of-phase for remainder of the template.[22] Another shortcoming is that short read lengths tend to produce biased sequence coverage that occurs in AT-rich repetitive sequences.[38]

APPLIED BIOSYSTEMS SOLiD:

The SOLiD (Supported Oligonucleotide Ligation and Detection) System 2.0 platform is a short-read sequencing technology based on ligation. This approach was developed in the laboratory of George Church and reported in 2005 along with resequencing of Escherichia coli genome.[121] This system

can generate 4 GB of sequence but the reads are only 35 nucleotides.[121] The weakness of SOLiD system is that it yields biased sequence coverage in AT-rich repetitive sequences[38] and only 35% of the raw reads are useable, compared with 95% for the 454 system. Another disadvantage is that it requires long run times.

THE HUMAN ORAL MICROBIOME DATABASE

Research over the past 20 years has focused on defining breadth and diversity of oral microbiome by obtaining 16S rRNA gene sequence information for both cultivable and as yet uncultivated oral bacteria.[113] The majority of bacterial species isolated from the oral cavity are included in 4 of the 10 bacterial phyla; Phylum 1 (Proteobacteria), Phylum 2 (the gram-positives), Phylum 5 (the spirochetes) and Phylum 6 (the flavobacter-bacteroides group).There are no known human oral representatives from the other 6 phyla. Though human oral microbiome is the most studied human microflora, 53% of species have not been named yet and 35% of species are uncultivated. The uncultivated taxa are identified mainly by 16S rRNA sequence information. (Figure 4; Figure 5)

The ***Human Oral Microbiome Database (HOMD)*** is a specifically designed database to provide a provisional naming scheme where each oral taxon is given a human oral taxon (HOT) number linked to comprehensive information and tools for examining and analyzing each taxon in the human oral microbiome at both taxonomic and genomic level.[17] This dynamic database provides a curated taxonomy of oral prokaryotes, a curated set of

full-length 16S rRNA reference sequences, and BLAST tools that allow identification of unknown isolates or clones based on their 16S rRNA sequence; additionally phenotypic, bibliographic, clinical and genomic information are linked for each taxa. Organisms of the human oral cavity are organized in a taxonomy hierarchy, which leads to individual pages for every oral taxon with comprehensive information and links. The genomic component of HOMD contains both static and dynamically updated annotations as well as bioinformatics analysis tools for all the genomic sequences, and curated 16S rRNA gene reference sequences for all human oral microbes. HOMD may serve as an example of a body site-specific tool for other communities.

More recently, a similar database was set up by **Griffen A et al**[31] known as CORE, a phylogenetically curated 16S rDNA database of the core oral microbiome, which offers improved and more robust identification of human oral bacterial 16S rRNA gene sequences compared with other methods. Its main goal is to provide a comprehensive and minimally redundant collection of oral bacteria at the genus and species level, as well as providing support for inferring community divergence and analysis of large datasets.

The basic list of oral bacteria came from the literature works of **Dzink JL**[25,26]**, Sockransky**[106]**, Tanner**[112,113] and **Moore WE**[79]. In 2010, **Dewhirst** identified 1,179 taxa of which 24% were named, 8% were cultivated but unnamed, and 68% were uncultivated phylotypes.[54] Upon validation, 434 novel non-singleton taxa were added to the HOMD. **Liu B et al**[63] reported using a

metagenomic approach by applying next-generation sequencing to sequence entire microbial DNA within a sample directly, and reconstructing genomes of microbiota via de novo assembly or mapping against a reference genome database. According to **Blainey P[14],** the emerging field of single-cell genomics is also currently being implemented for bacteria and Archaea. The issue with these commercially available tests is the question of their true value in terms of reliability for detecting causative agents of disease, given our limited knowledge of the complex ecosystem involved. The other major concern lies in the ability of clinician or diagnostic company to interpret results correctly and in such a way as to provide benefit for patients.[21]

STUDIES ON THE ANALYSIS OF PLAQUE MICROBIOME

The proven microbiological etiology of periodontal diseases is the rationale for application of various methods for microbiological identification, in order to accomplish better diagnosis and for targeted anti-infectious periodontal treatment.[53] Various microorganisms inhabiting the periodontal environment and their complex relationships were presented by Socransky and Haffajee and were united in periodontal complexes.

The first complete sequence of a microbial genome was published in **1995** by **Fleischmann RD et al**.[28] In the past 50 years, numerous studies by **Paster et al[87], Baker et al[9], Kumar et al[54] and Aas et al[2]** have characterized the community composition of oral microbiota.. Using culture-dependent and independent methods, estimates of oral biodiversity have implicated more than 700 different microbial species.[2,87,105] The composition of subgingival

microbiota of chronic periodontitis in adults has been described by culture[79,112], immunological and molecular methods.[54,105] Culture analysis of subgingival plaque samples of early periodontitis by **Tanner et al**[112] detected *Tannerella forsythia, Campylobacter rectus, and Selenomonas noxia* associated with progressing disease pattern compared with non-progressing disease subjects, whereas *Porphyromonas gingivalis* was associated, by whole genomic DNA probes, with progressing periodontitis. These species have also been associated with moderate and advanced periodontitis.[79,105] Molecular PCR cloning and sequencing methods have identified several species that are rarely or not detected by culture methods,[87] some of which show strong associations with adult periodontitis.[54] **Aas J et al**[2] in a study in **2005** established that there is a distinctive predominant bacterial flora of the healthy oral cavity that is highly diverse, as well as site and subject specific. They found 141 predominant species of which 60% have not been cultivated. 13 new phylotypes were identified, and species typically associated with periodontitis were not detected.

Tanner AC et al[113] conducted a cross-sectional evaluation of 141 healthy and periodontitis individuals to compare microbiota of subgingival and tongue samples between early periodontitis and health using oligonucleotide probes and PCR. *Porphyromonas gingivalis and Tannerella forsythia* were associated with early periodontitis by direct PCR, and they found that microbiota of tongue samples was less sensitive than that of subgingival samples in detecting periodontal species.

Several studies have employed next-generation sequencing technologies to analyze the species richness of the oral microbiota.[46,132]. Estimates from one of these studies by **Keijser et al (2008)**[46] suggested that up to 19,000 phylotypes may exist in human oral cavity. Keijser conducted a study for pyrosequencing analysis of oral microflora from saliva and supragingival plaque in 71 and 98 healthy individuals respectively using 454 Life sciences and Genome Sequencer 20 system. His results generated 19,000 phylotypes representing 22 taxonomic phyla and 3621 and 6888 species-level phylotypes in saliva and plaque respectively. He showed that the vast majority (namely 99.6%) of sequences in saliva and subgingival plaque samples of adults belong to one of the seven major phyla: *Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes, or candidate division TM7*[46].

G Xie et al[128] reported a metagenomic analysis of a healthy human plaque sample using a combination of second generation sequencing platforms, and revealed the presence of 12 well-characterized phyla, members of the TM-7 and BRC 1 clade, and unclassified sequences. 73% of the total assembled contig sequences were predicted to code for proteins, 2.8% of the predicted genes coded for proteins involved in resistance to antibiotics and toxic compounds.

Liu B et al[63] performed a pilot study in 2012 to analyze the global genetic, metabolic and ecological changes associated with periodontitis in 15 subgingival plaque samples from two periodontitis patients and three healthy

individuals using metagenomics. They found that the disease samples shared a similar bacterial species cluster that was different from the completely healthy samples suggesting that the disease state occupied a narrow region within the space of possible configurations of the oral microbiome. They observed a shift in the oral bacterial composition from a gram-positive dominated community in the healthy subject to a gram-negative dominated community in periodontal disease. The shift in bacterial species from gram-positive to gram-negative confirmed previous findings using different molecular biological methods. Liu and colleagues also observed higher bacterial diversity in the diseased samples than in the healthy samples, which confirmed results obtained using 16S rRNA sequence analysis.

Griffen et al[32] conducted a study using 454 pyrosequencing of 16S rRNA genes and identified and reported 16 phyla, 106 genera and 596 species. Community diversity was higher in disease, 123 species were significantly abundant in disease and 53 species in health. *Spirochaetes, Synergistetes and Bacteroidetes* were more abundant in disease whereas *Proteobacteria* were found in higher levels in healthy controls. Within the phylum *Firmicutes*, the class Bacilli was health associated whereas *Clostridia, Negativicutes and Erysipelotrichia* were associated with disease.

Abusleme et al[3] conducted a study in 2013 to and found a higher biodiversity and biomass in periodontitis compared to health, with periodontitis having higher proportions of *Spirochetes, Synergistetes, Firmicutes and Chloroflexi;* while the proportions of *Actinobacteria* like

Actinomyces were increased in health. They also showed an association between biomass and community structure in periodontitis with proportions of specific taxa correlating with bacterial load.

Trajanoski et al[117] conducted a study to demonstrate pyrosequencing data processing for the characterization of the subgingival microbiome in healthy children and reported 2617 operational taxonomic units (OTU) that were classified into 11 phyla with the majority accounted for by *Bacteroidetes* (27.24%), *Actinobacteria* (14.21%), *Firmicutes* (17.92%), *Proteobacteria* (10.85%), *Spirochaetes* (4.09%) and *Fusobacteria* (3.59%) in the subgingival samples.

Wang J and colleagues[124] they analyzed periodontal samples and found a strong correlation between bacterial community structure and disease status, and identified numerous novel microbial inhabitants. The 4 most abundant phyla were *Bacteroidetes* (41-59.2%), *Actinobacteria* (9.3-41%), *Proteobacteria* (5.2-40.1%) and *Firmicutes* (14.8-58.3%). They also examined FimA type, an important biofilm gene involved in interactions of *Porphyromonas gingivalis* with other microorganisms. They found that the most prevalent P. gingivalis FimA was type II, which is consistent with previous studies.

Xiuchun Ge et al[30] examined the subgingival bacterial biodiversity in 88 untreated chronic periodontitis patients by comparing the oral microbiome in deep (diseased) and shallow (healthy) sites. 51 of 170 genera and 200 of 746 species were found significantly different in abundances between the 2

sites. They also reported that this difference was influenced by patient level effects such as clinic location, race and smoking.

In a study by **Yan Li et al**[60] they examined the phylogenetic and functional gene differences between 25 periodontal and 12 healthy subjects using MiSeq sequencing. 39 genera were significantly different between healthy and periodontitis group and *Fusobacterium, Porphyromonas, Treponema, Filifactor, Eubacterium, Tannerella, Hallella, Parvimonas, Peptostreptococcus and Catonella* showed higher relative abundances in the periodontitis groups. A variety of genes involved in virulence factors, amino acid metabolism and glycosaminoglycan and pyrimidine degradation were enriched in periodontitis whereas genes involved in amino acid synthesis and pyrimidine synthesis exhibited a significantly lower relative abundance compared with healthy group.

Shi B et al[102] aimed to determine whether dynamic changes in the subgingival microbiome in periodontitis patients before and after treatment at the same tooth sites can serve as a diagnostic and prognostic indicator. 38 genera that had an abundance of more than 1% were identified, *Prevotella* and *Fusobacterium* being the most abundant genera. Their results suggested that *Synergistetes, Filifactor and Mycoplasma* should be considered expanded members of the red complex.

Hong BY et al[42] conducted a study to explore the existence of different community types in periodontitis and their relationship with host demographic, medical and disease-related clinical characteristics. Their results

suggested 2 types of communities (A and B) existed in periodontitis. Type B communities harbored greater proportions of certain periodontitis associated taxa like *P. gingivalis, T. forsythia and T. denticola* and other recently linked periodontitis associated ones. In contrast, type A communities had increased proportions of different species and were also enriched for health associated species and core taxa.

Zheng et al[135] performed a study to analyze the microbial characteristics of oral plaque around implants using pyrosequencing of 16S rRNA gene, and reported an increase in microbial diversity in subgingival sites of ailing implants compared with healthy implants. Periodontal pathogens like *P. gingivalis, T. forsythia and P. intermedia* were clustered into modules in the peri-implant mucositis network (**Zheng H et al, 2015**).

Kotsilkov (2015)[53] compared the diagnostic potential of microbiologic culture and real-time PCR identification for detection of putative periodontopathic bacteria of 60 patients from deep periodontal pockets with probing depth of more than 7mm. Their results yielded a statistically significant higher detection levels and better diagnostic capability of the RT-PCR, whereas the culture analysis detected only 3 pathogens (**Kotsilkov K et al, 2015**).

Mason MR et al (2015)[73] studied the subgingival microbiome of clinically healthy current and never smokers to assess the extent to which smoking can increase risk for periodontal disease by influencing subgingival microbiome composition. Smokers demonstrated a highly diverse, pathogen

rich, commensal poor, anaerobic microbiome that was closely aligned with disease associated communities in clinically healthy individuals.

Payungporn et al (2017)[88] conducted a study to identify potential bacterial species associated with periodontal disease in ten Thai patients within the age group of 43 to 53 years, of which 5 were from healthy controls and 5 were patients with chronic periodontitis. It was observed that *P. gingivalis* and *P. intermedia* were significantly associated with periodontal disease, whereas other bacteria like *T. denticola, T. medium, Tannerella forsythia, P. endodontalis and F. alocis* may be potentially associated with periodontal disease in Thai patients.

Santigli E et al (2017)[99] conducted a study in children to study the sampling modification effects in the subgingival microbiome profile of healthy children. The 5 major phyla found in all samples were *Actinobacteria, Bacteroidetes, Proteobacteria, Firmicutes and Fusobacteria*.

FIGURE 1: BACTERIAL COMPLEXES

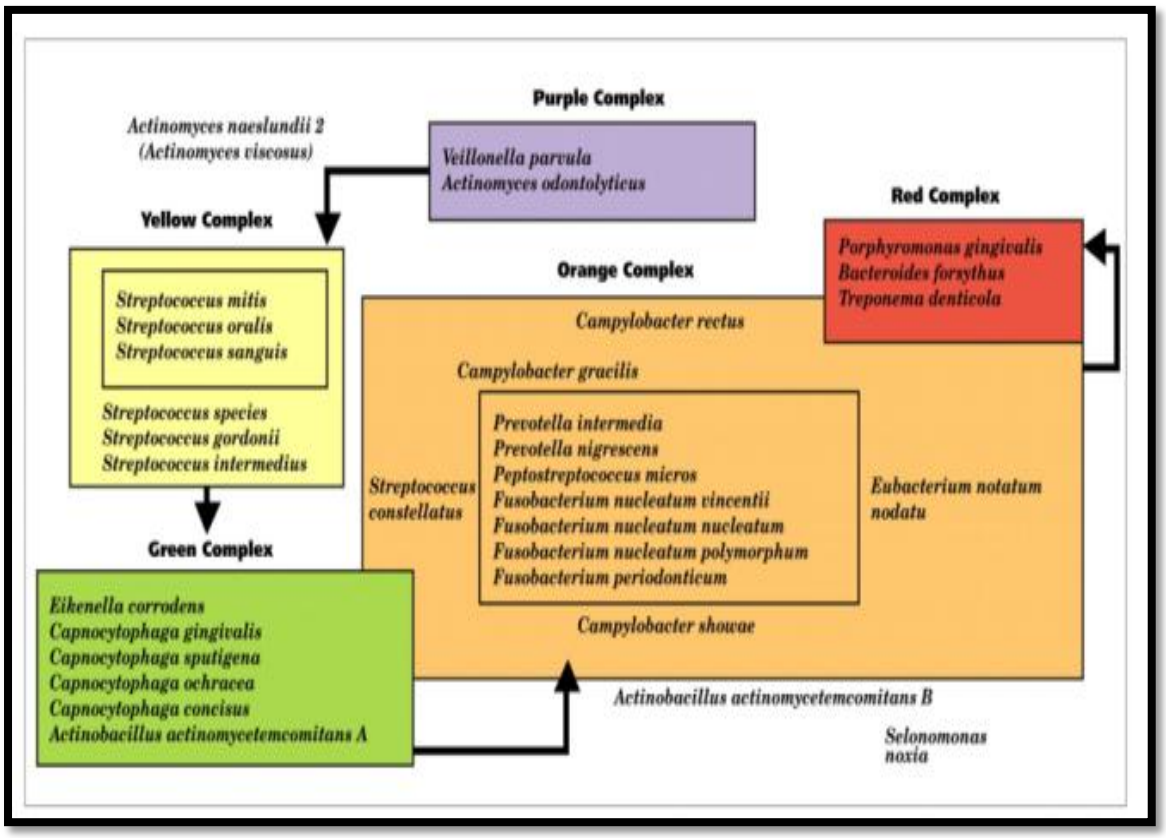


FIGURE 2: ROCHE 454 LIFE SCIENCES SYSTEM

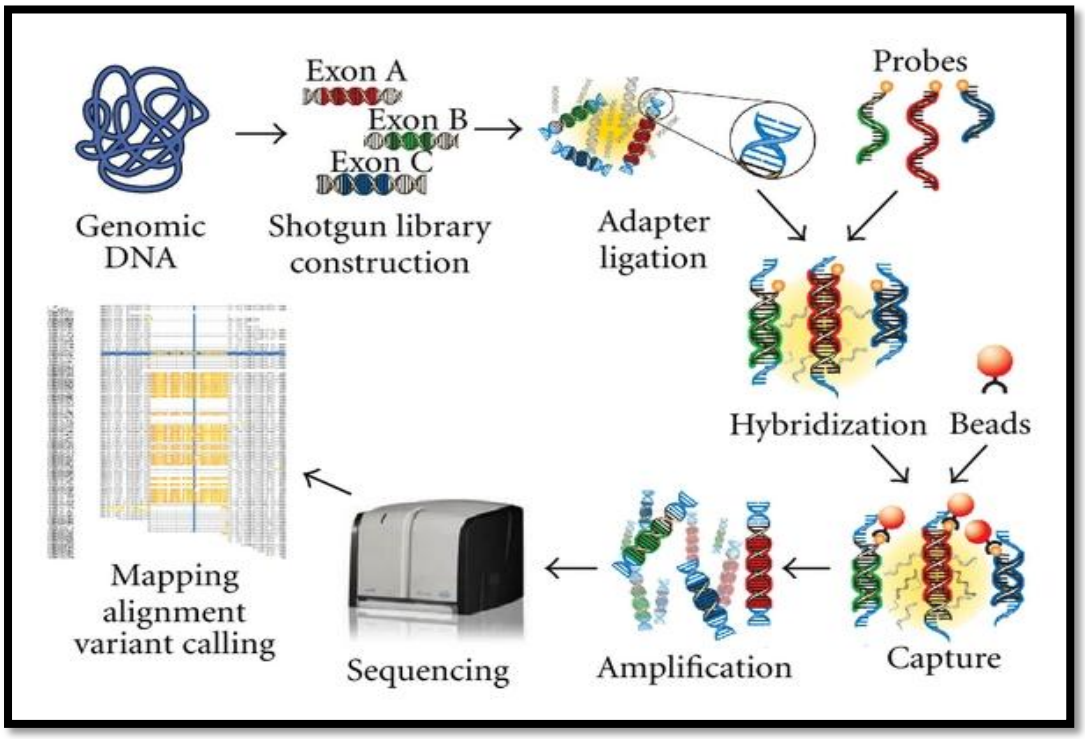


FIGURE 3: ILLUMINA SOLEXA GENOME ANALYZER

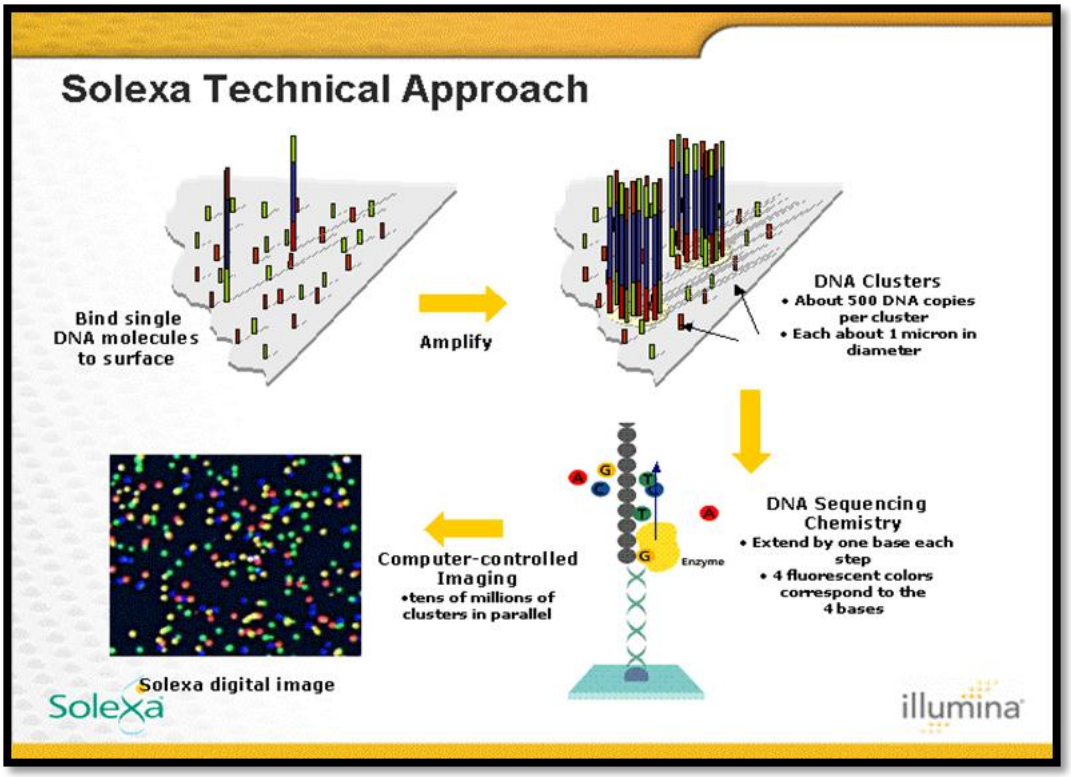
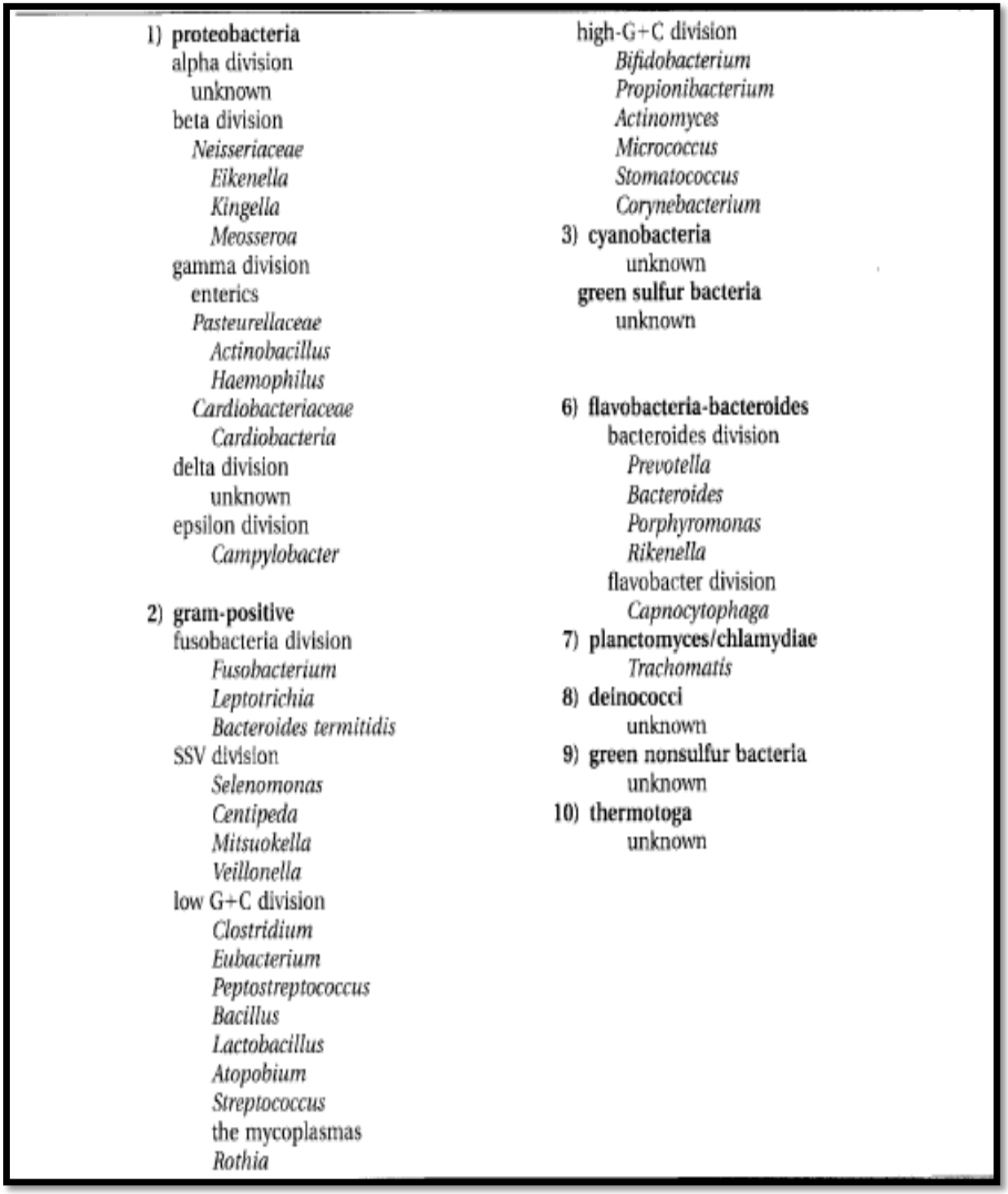


FIGURE 4: TAXONOMY OF ORAL BACTERIA (10 MAJOR PHYLA)



1) **proteobacteria**
- alpha division
  - unknown
- beta division
  - *Neisseriaceae*
    - *Eikenella*
    - *Kingella*
    - *Meosseroa*
- gamma division
  - enterics
  - *Pasteurellaceae*
    - *Actinobacillus*
    - *Haemophilus*
  - *Cardiobacteriaceae*
    - *Cardiobacteria*
- delta division
  - unknown
- epsilon division
  - *Campylobacter*

2) **gram-positive**
- fusobacteria division
  - *Fusobacterium*
  - *Leptotrichia*
  - *Bacteroides termitidis*
- SSV division
  - *Selenomonas*
  - *Centipeda*
  - *Mitsuokella*
  - *Veillonella*
- low G+C division
  - *Clostridium*
  - *Eubacterium*
  - *Peptostreptococcus*
  - *Bacillus*
  - *Lactobacillus*
  - *Atopobium*
  - *Streptococcus*
  - the mycoplasmas
  - *Rothia*
- high-G+C division
  - *Bifidobacterium*
  - *Propionibacterium*
  - *Actinomyces*
  - *Micrococcus*
  - *Stomatococcus*
  - *Corynebacterium*

3) **cyanobacteria**
- unknown

green sulfur bacteria
- unknown

6) **flavobacteria-bacteroides**
- bacteroides division
  - *Prevotella*
  - *Bacteroides*
  - *Porphyromonas*
  - *Rikenella*
- flavobacter division
  - *Capnocytophaga*

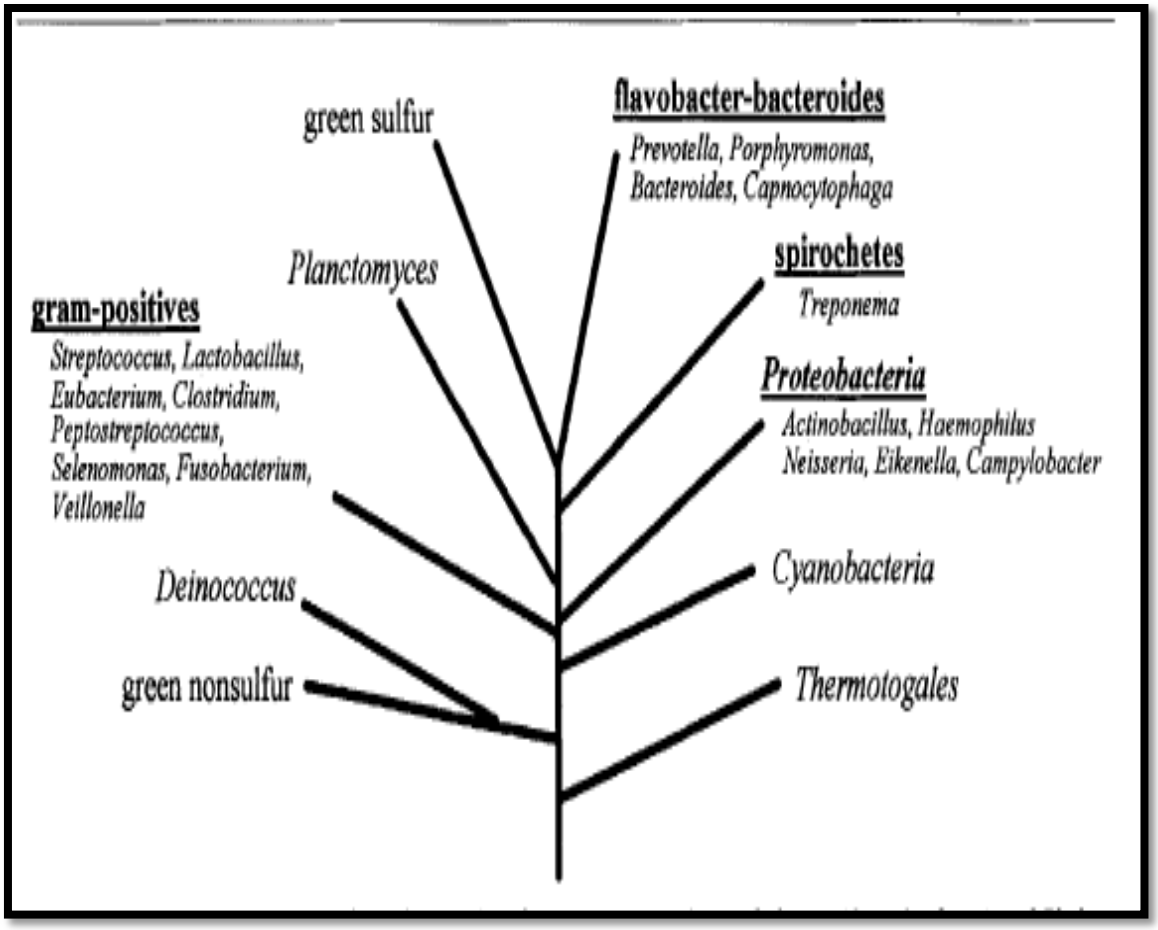
7) **planctomyces/chlamydiae**
- *Trachomatis*

8) **deinococci**
- unknown

9) **green nonsulfur bacteria**
- unknown

10) **thermotoga**
- unknown

FIGURE 5: PHYLOGENETIC TREE ILLUSTRATING THE 10 BACTERIAL PHYLA, BASED ON 16S rRNA SEQUENCE COMPARISONS



Materials and Methods

MATERIALS AND METHODS

Study population

Microbiome studies are arduous to perform in large populations owing to intricacy of the technology used, magnitude of data obtained and cost involved. This study comprised of a small population of eight subjects, in line with earlier studies by **Zheng et al**[105] and **Dzink et al**.[25]

Individuals seeking dental treatment in Ragas Dental College and Hospitals, Chennai, were included in the present study. Certificate of ethical clearance for the study was obtained from Institutional Review Board of Ragas Dental College. Eight subjects were recruited and segregated into two groups; comprising four periodontally healthy subjects as controls and four subjects with gingival recession diagnosed as localized chronic periodontitis as test group. Diagnosis of health and disease was determined based on the parameters recommended by American Academy of Periodontology.[126]

Control Group comprised four subjects with clinically non-inflamed, healthy gingiva having probing pocket depth (PPD) ≤3mm, no clinical attachment loss {CAL}, no bleeding on probing {BOP}.

Test Group comprised four patients exhibiting gingiva recession in at least 1 site, with presence of local factors and clinical signs of inflammation, having probing pocket depth ≤3mm and clinical attachment loss ≥5mm.

The study protocol was explained to participants and written informed consent was obtained from each individual before clinical periodontal

examination and subgingival plaque sampling. Medical and dental histories were obtained.

INCLUSION CRITERIA

- Subjects exhibiting good systemic health
- Subjects fulfilling the criteria of periodontal health and disease as described above were included in the study

EXCLUSION CRITERIA

- Patient with systemic disorders, such as diabetes mellitus or immunological disorders, HIV
- Patients on drugs that have potential to interfere with microbial characteristics such as immunosuppressant drugs or steroids.
- Patients with history of tobacco usage
- Patients with history of periodontal treatment in the past 6 months
- Patients under antimicrobial therapy for the past 6 months

SUBGINGIVAL PLAQUE SAMPLING

All examinations were performed by a single examiner using calibrated periodontal probe. In test group, supragingival plaque was first removed from sample teeth with a sterile Gracey curette. The site was then cleaned and isolated using cotton rolls and air dried gently. Another sterile Gracey curette was inserted subgingivally into recession site and plaque was

removed by applying a slight stroking force towards the coronal aspect of the tooth. Tip of the curette was then inserted into Eppendorf tube containing ionized molecular water and shaken until the plaque was removed from the curette. For healthy subgingival plaque samples, sites that did not exhibit any signs of inflammation and bleeding on probing were chosen. The same procedure was followed for obtaining subgingival samples from these sites.

The samples obtained were frozen and stored at -20°C until sample collection period was completed. Sample collection was completed within 2 days and samples were sent for processing so as to avoid any proteolytic degradation of the components.

DNA EXTRACTION, 16S rRNA AMPLIFICATION, LIBRARY CONSTRUCTION AND SEQUENCING

Genomic DNA was extracted from the eight subgingival plaque samples with the Fast DNA kit and the FastPrep 24-5G instrument according to manufacturer's recommendations (MP Biomedicals, Santa Ana, CA). Extracted DNA was purified with silica-based spin filters (FastDNA kit) and DNA was amplified using the 16S V3 (341F) forward and V4 (805R) reverse primer pairs with added Illumina adapter overhang nucleotide sequences.

The amplicon generation was done using PCR to amplify a template out of a DNA sample using region-of-interest specific primers with overhang adapters attached. Amplicon synthesis was performed using thermocycling with 2.5µl of microbial DNA (5ng/ µl), 5µl of amplicon PCR forward primer (1µM), 5µl of amplicon PCR reverse primer (1µM), and 12.5µl of 2x KAPA

HiFi HotStart Ready Mix (Kapa Biosystems) at 95°C initial denaturation for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

Reactions were cleaned up with Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer's protocol. Attachment of dual indices and Illumina sequencing adapters was performed using 5µl of amplicon PCR product DNA, 5µl of Illumina Nextera XT Index 1 Primer (N7xx) from the Nextera XT Index kit, *5* µl of Nextera XT Index 2 Primer (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10µl of PCR-grade water (UltraClean DNA-free PCR water; MO BIO Laboratories, Inc., Carlsbad, CA, USA), with thermocycling at 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

Constructed 16S metagenomic libraries were purified with Agencourt AMPure XP beads and quantified using a fluorometric quantification method that uses dsDNA binding dyes with Quant-iT PicoGreen and the KAPA Library Quantification Kit (KAPABIOSYSTEMS). Library quality control was performed with the Agilent Technologies 2100 Bioanalyzer to ascertain quality and average size distribution.

Samples were denatured and diluted to a final concentration of 10 pM with a 20% PhiX (Illumina) control. Sequencing was performed using the Illumina Miseq System. After samples are loaded, the MiSeq system provides on instrument secondary analysis using the MiSeq Reporter software (MSR).

All eight samples were multiplexed and sequenced in a single lane on the MiSeq using 2 × 300 bp paired-end sequencing. Operational taxonomic units (OTUs) were assigned to each sequence using Human Oral Microbiome Database. The Metagenomics workflow classified organisms from the V3 and V4 amplicon using a database of 16S rRNA data, and this classification is based on the Greengenes database. The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. The analysis output is represented as Bar Graphs, Tables and Cluster Pie Charts.

Statistical analysis

Data was analyzed as recommended by earlier studies.[32,54,63] Data obtained was compiled systematically in Microsoft excel spreadsheet, dataset was subdivided and distributed meaningfully and presented as graphs and tables.

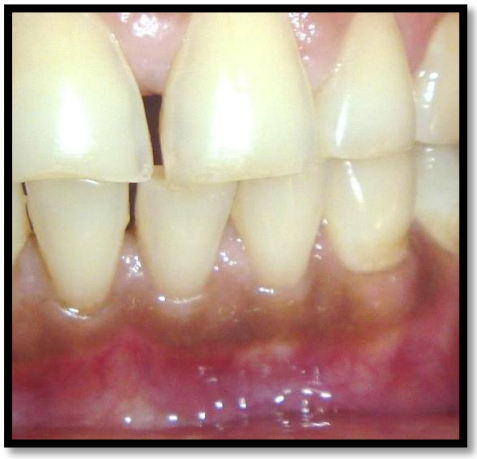
Statistical analysis was performed using Statistical Package of Social Sciences software (IBM Corp. Released 2011. SPSS Statistics for Windows, version 20.0 Armonk, NY: IBM Corp). Normality test was done using Kolmogorov-Smirnov test and Shapiro-Wilk numerical test, and it was found that all variables were normally distributed. Depending on the nature of data appropriate parametric statistical tests were chosen. P value of <0.05 was considered to be significant.

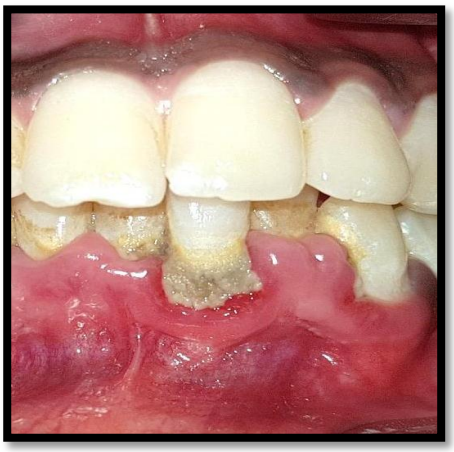
Student's unpaired t-test was used to compare mean relative abundance of 20 different species in health with that in gingival recession, and vice versa.

Circular maximum likelihood phylogenetic tree at the level of genus was constructed using iTOL and PhyloT tools as reported in earlier studies by **Griffen et al**.[32]

Photographs

HEALTHY SITE **GINGIVAL RECESSION SITE**

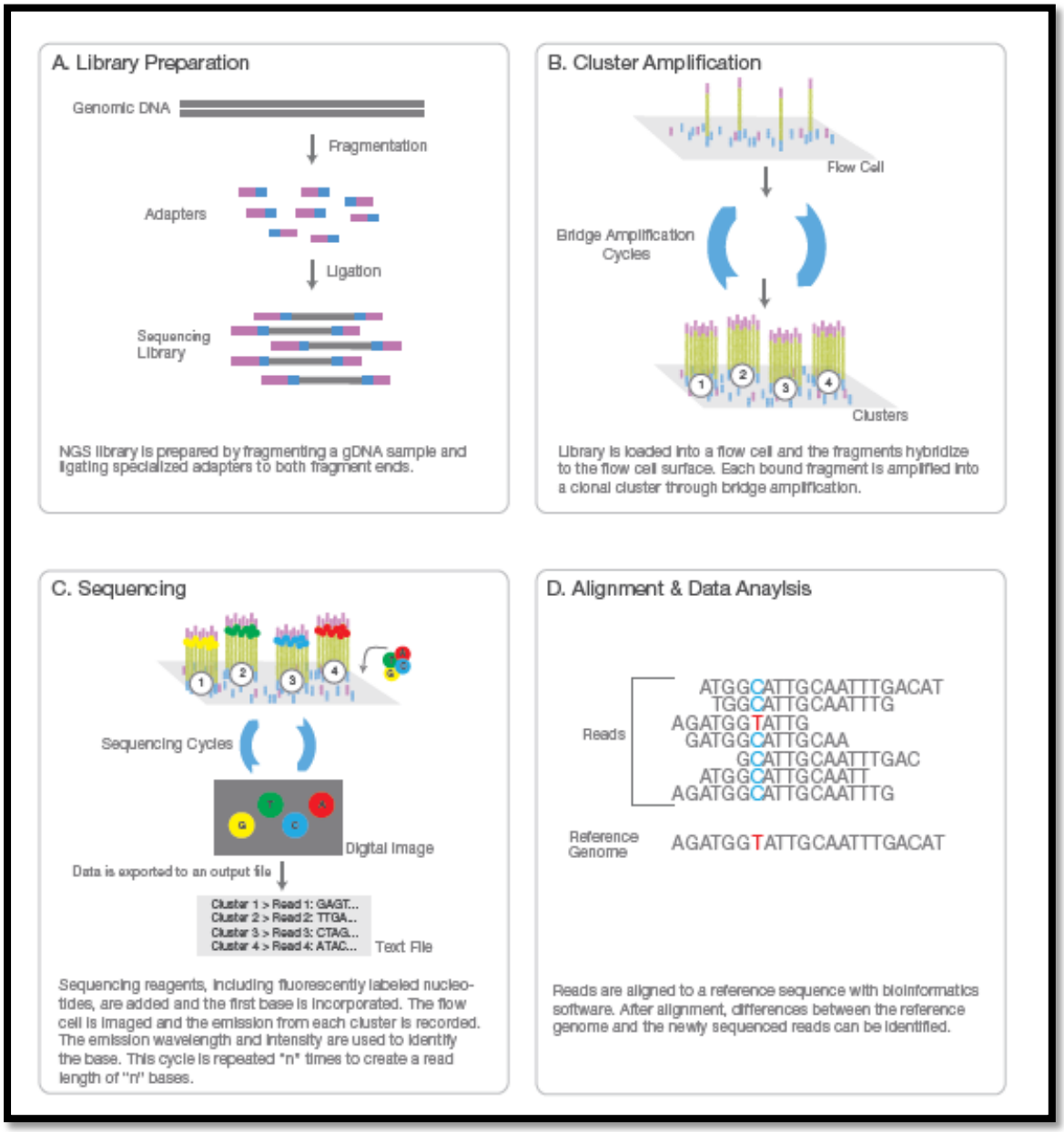




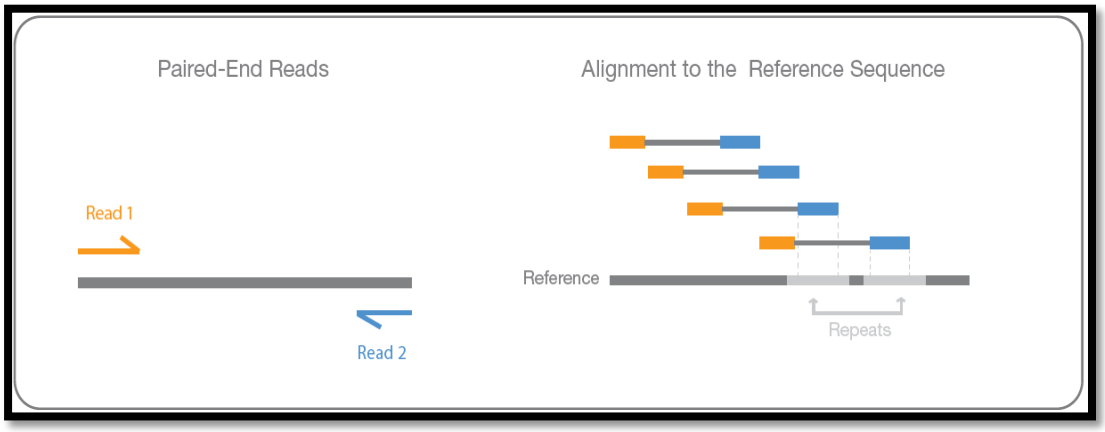
SAMPLE COLLECTION AND STORAGE

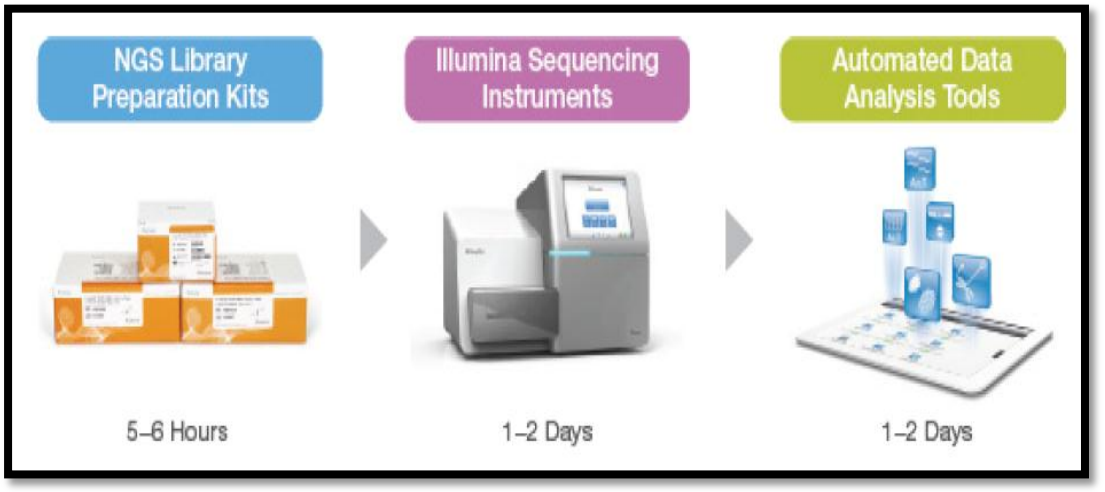
ILLUMINA SEQUENCING

FOUR BASIC STEPS IN ILLUMINA NGS WORK FLOW

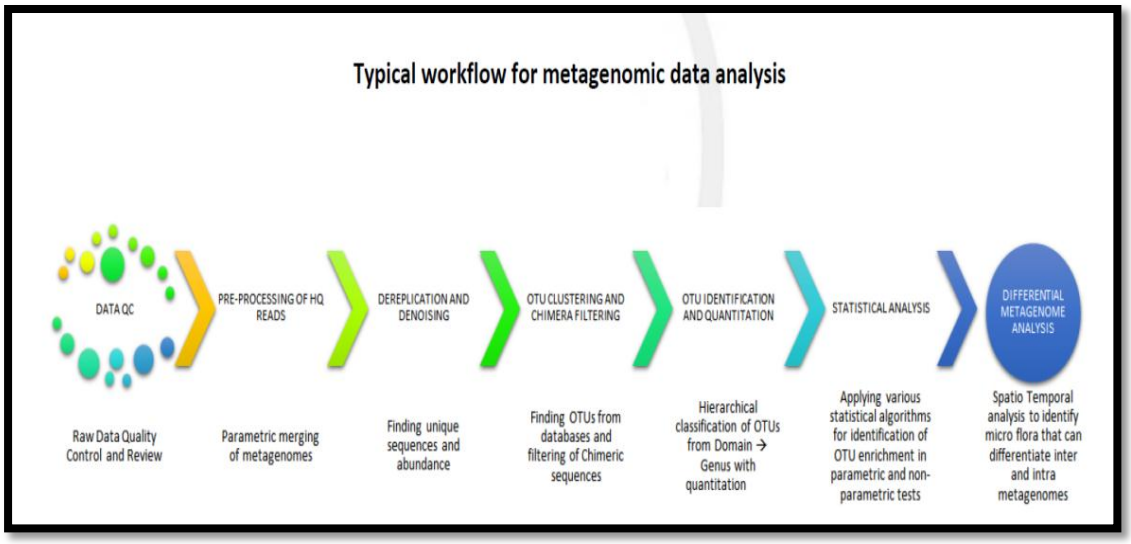


PAIRED-END SEQUENCING AND ALIGNMENT





WORKFLOW FOR METAGENOMIC DATA ANALYSIS



Results

RESULTS

The present study was carried out among a small population of eight individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai. The age distribution of the study participants ranged from 20-53 years with a mean age of 32.66 years.

Subgingival plaque samples were collected using sterile Gracey curettes from four periodontally healthy control individuals (designated as H1, H2, H3 and H4) and from gingival recession sites of four individuals with localized chronic periodontitis (designated as R1, R2, R3 and R4).

Amplicons from V3-V4 hypervariable regions of 16S rRNA gene were sequenced. The results obtained are represented according to taxonomic classification system of bacteria, and individual comparisons are depicted through tables and graphs.

PHYLOGENETIC COMPOSITION OF MICROBIOME

Distribution of bacteria in healthy and gingival recession sites:

The distribution of bacteria in sites of health and gingival recession at phylum, genus and species level is shown in Table 1 and Graph 1-A, 1-B and 1-C. In the healthy sites, a total of 27 phyla, 558 genera and 1063 species; and in gingival recession sites 29 phyla, 641 genera and 1279 species have been identified.

Top 5 phyla in healthy and gingival recession sites:

The top 5 phyla in healthy and gingival recession sites along with their respective abundances are reported in Table 2 and Graph 2-A and 2-B. In healthy sites, phylum Firmicutes has shown highest abundance of 15.75% followed by Proteobacteria (14.07%), Bacteroidetes (11.23%), Fusobacteria (6.77%) and Cyanobacteria (1.25%). In gingival recession sites, phylum Proteobacteria has shown highest abundance of 15.69% followed by Bacteroidetes (15.55%), Firmicutes (10.66%), Fusobacteria (10.01%) and Actinobacteria (0.90%).

Top 5 genera in healthy and gingival recession sites:

The top 5 genera in healthy and gingival recession sites along with their respective abundances have been reported in Table 3 and Graph 3-A and 3-B. In both healthy and gingival recession sites, Fusobacterium is the most abundant genus to be identified with an abundance of 9.18% and 5.52% respectively. Following Fusobacterium, the next four genera in healthy sites are Campylobacter (5.24%), Capnocytophaga (4.58%), Chryseobacterium (2.40%) and Porphyromonas (1.95%) and in gingival recession sites are Campylobacter (4.68%), Porphyromonas (3.58%), Treponema (3.45%) and Chryseobacterium (3.40%).

Comparison of subgingival microbiome in healthy versus gingival recession sites at genus level: phylogenetic tree

The subgingival microbiome was compared between healthy and gingival recession sites at genus level and is represented in the form of a

circular phylogenetic tree in Graph 3-C. The tree has been constructed with phyloT software and is displayed using iTOL (**Letunic and Bork, 2011**). The bars in the outer band (orange) represent the relative abundance of bacterial genera in healthy (blue) and gingival recession (green) sites.

Health-associated and disease-associated microbiome:

Among a total of 1063 and 1279 species that have been identified in healthy and gingival recession sites respectively, 900 species are commonly seen in both groups; 161 species and 381 species are uniquely present only in healthy sites and in gingival recession sites respectively. These organisms have been listed out in table 4 and table 5.

Relative abundance of top 20 bacterial species in healthy versus gingival recession sites:

Table 6 depicts relative abundance of top 20 bacterial species in healthy versus recession sites.. Among species in health sites, *Fusobacterium naviforme* exhibited maximum abundance of 4.89% and *Neisseria lactamica* exhibited minimum abundance of 0.44%.

The statistical difference between health and gingival recession sites were found to be highly significant ($p<0.001$) in case of *Fusobacterium periodonticum* ($p=0.000$) and *Capnocytophaga gingivalis* ($p=0.001$); significant in case of *Zhouia amylolytica* ($p=0.034$), *Capnocytophaga leadbetteri* ($p=0.019$), *Fusobacterium gonidiformins* ($p=0.010$) and *Capnocytophaga ochracea* ($p=0.040$); and non-significant in case of all other species (Graph 4).

Relative abundance of species in health among top 20 species in gingival recession:

Table 7 compares relative abundance of top 20 bacterial species of gingival recession sites with their corresponding abundance percentage in healthy samples. Among species in gingival recession sites, *Fusobacterium naviforme* had maximum abundance of 3.69% and *Neisseria mucosa* had minimum abundance of 0.37%.

The statistical difference between top 20 species in recession samples and corresponding health samples were found to be significant in case of *Zhouia amylolytica* (p=0.034) and non-significant in all other species (Graph 5).

Tables and Graphs

TABLE 1: DISTRIBUTION OF BACTERIA IN HEALTHY AND GINGIVAL RECESSION SITES

CRITERIA	PHYLUM	GENUS	SPECIES
HEALTH	27	558	1063
RECESSION	29	641	1279

TABLE 2: TOP 5 PHYLA IN HEALTHY AND GINGIVAL RECESSION SITES

S.No	HEALTH	ABUNDANCE (%)	GINGIVAL RECESSION	ABUNDANCE (%)
1	Firmicutes	15.75	Proteobacteria	15.69
2	Proteobacteria	14.07	Bacteroidetes	15.55
3	Bacteroidetes	11.23	Firmicutes	10.66
4	Fusobacteria	6.77	Fusobacteria	10.01
5	Cyanobacteria	1.25	Actinobacteria	0.90

TABLE 3: TOP 5 GENERA IN HEALTHY AND GINGIVAL RECESSION SITES

S.No	HEALTH	ABUNDANCE (%)	GINGIVAL RECESSION	ABUNDANCE (%)
1	Fusobacterium	9.18	Fusobacterium	5.52
2	Campylobacter	5.24	Campylobacter	4.68
3	Capnocytophaga	4.58	Porphyromonas	3.58
4	Chryseobacterium	2.40	Treponema	3.45
5	Porphyromonas	1.95	Chryseobacterium	3.40

TABLE 4: PERIODONTAL HEALTH-ASSOCIATED MICROBIOME

(161 species- present in health, not in disease)

	A		
1	Acidovorax caeni	8	Acidithiobacillus cuprithermicus
2	Acinetobacter antiviralis	9	Acidovorax temperans
3	Actinocorallia herbida	10	Acinetobacter seohaensis
4	Agromyces rhizospherae	11	Agromyces mediolanus
5	Aneurinibacillus danicus	12	Anaerolinea thermophila
6	Avibacterium paragallinarum	13	Arcanobacterium haemolyticum
7	Azomonas macrocytogenes	14	Azomonas insignis
		15	Azorhizophilus paspali
	B		
16	Bacillus methanolicus	18	Bifidobacterium choerinum
17	Burkholderia brasilensis	19	Burkholderia ginsengisoli
		20	Burkholderia phenazinium
	C		
21	Campylobacter subantarcticus	28	Caldisericum exile
22	Candidatus fragariae	29	Candidatus Azobacteroides
23	Caulobacter crescentus	30	Candidatus prunorum
24	Cellulophaga fucicola	31	Caulobacter tundra
25	Clostridium straminisolvens	32	Chitinophaga soli
26	Comamonas denitrificans	33	Collimonas pratensis
27	Corynebacterium doosanense	34	Comamonas terrigena
		35	Cupriavidus pinatubonensis
	D		
36	Dactylosporangium vinaceum	43	Deinococcus piscis
37	Deinococcus yavapaiensis	44	Delftia lacustris
38	Denitrobacter permanens	45	Dermacoccus barathri
39	Dermacoccus profundi	46	Desulfofrigus oceanense
40	Desulfovibrio piger	47	Desulfovibrio simplex
41	Dialister invisus	48	Dokdonella fugitive
42	Dolichospermum curvum	49	Dysgonomonas hofstadii

	E		
50	Ectothiorhodospira haloalkaliphila	54	Eikenella corrodens
51	Elizabethkingia anophelis	55	Elizabethkingia meningoseptica
52	Enterococcus durans	56	Enterococcus faecalis
53	Enterococcus hermanniensis	57	Enterococcus italicus
		58	Entomoplasma somnilux
	F		
59	Friedmanniella capsulata	60	Flavobacterium denitrificans
		61	Fusobacterium necrophorum
	G		
62	Gemella morbillorum	64	Geovibrio thiophilus
63	Gluconobacter krungthepensis	65	Gluconobacter morbifer
		66	Gramella marina
	H		
67	Halomonas johnsoniae	70	Hahella antarctica
68	Hydrogenophaga intermedia	71	Herbaspirillum aquaticum
69	Hymenobacter gelipurpurascens	72	Hydrogenophaga pseudoflava
	K		
73	Kushneria aurantia	74	Knoellia aerolata
	L		
75	Lactobacillus letivazi	76	Lactobacillus brantae
	M		
77	Marinomonas pontica	82	Marinobacter szutsaonensis
78	Microbacterium halophilum	83	Methyloversatilis universalis
79	Moraxella equi	84	Microvirgula aerodenitrificans
80	Mycoplasma adleri	85	Moraxella lacunata
81	Mycoplasma caviae	86	Mycoplasma agassizii
		87	Mycoplasma fastidiosum
	N		
88	Nitrincola lacisaponensis		
	O		
89	Ochrobactrum intermedium	90	Oceanisphaera donghaensis
	P		
91	Pasteurella eae	115	Paracoccus denitrificans
92	Pedobacter daejeonensis	116	Pasteurella pneumotropica
93	Pelagicoccus mobilis	117	Pedobacter himalayensis
94	Peptoniphilus ivorii	118	Pelomonas puraquae

95	Planomicrobium alkanoclasticum	119	Planctomyces maris
96	Pseudaminobacter defluvii	120	Propionibacterium acnes
97	Pseudomonas aeruginosa	121	Pseudoclavibacter helvolus
98	Pseudomonas amygdali	122	Pseudomonas alcaligenes
99	Pseudomonas citronellolis	123	Pseudomonas anguilliseptica
100	Pseudomonas fluorescens	124	Pseudomonas coronafaciens
101	Pseudomonas guineae	125	Pseudomonas fuscovaginae
102	Pseudomonas koreensis	126	Pseudomonas jinjuensis
103	Pseudomonas mandelii	127	Pseudomonas lundensis
104	Pseudomonas mendocina	128	Pseudomonas mediterranea
105	Pseudomonas monteilii	129	Pseudomonas metavorans
106	Pseudomonas mucidolens	130	Pseudomonas moraviensis
107	Pseudomonas orientalis	131	Pseudomonas nitroreducens
108	Pseudomonas otitidis	132	Pseudomonas oryzihabitans
109	Pseudomonas plecoglossicida	133	Pseudomonas pavonaceae
110	Pseudomonas pseudoalcaligenes	134	Pseudomonas poae
111	Pseudomonas resinovorans	135	Pseudomonas putida
112	Pseudomonas tolaasii	136	Pseudomonas stutzeri
113	Pseudomonas tropicalis	137	Pseudomonas tremae
114	Psychrobacter urativorans	138	Pseudomonas vancouverensis
		139	Psychromonas ossibalaenae
	R		
140	Roseomonas mucosa	141	Roseomonas terrae
		142	Rothia mucilaginosa
	S		
143	Salinicoccus salsiraiae	149	Saccharomonospora thermoviridis
144	Sanguibacter suarezii	150	Salinispora tropica
145	Shinella yambaruensis	151	Serratia entomophila
146	Streptococcus iniae	152	Steroidobacter denitrificans
147	Streptomyces flavoviridis	153	Streptococcus pseudoporcinus
148	Streptomyces sanglieri	154	Streptomyces matensis
		155	Streptomyces vitaminophilus
	T		
156	Thermodesulfovibrio aggregans	158	Thiobacillus sajanensis
157	Thiobacillus thiophilus	159	Thiorhodococcus mannitoliphagus
	V		**W**
160	Vagococcus carniphilus	161	Weissella minor

TABLE 5: DISEASE-ASSOCIATED MICROBIOME (381 species- present in disease, not in health)

	A				
		13	Acetobacterium submarinus	27	Acholeplasma equifetale
1	Acholeplasma hippikon	14	Acidisoma tundrae	28	Acidovorax valerianellae
2	Acinetobacter antiviralis	15	Acinetobacter beijerinckii	29	Acinetobacter psychrotolerans
3	Acinetobacter tjernbergiae	16	Actinoallomurus luridus	30	Actinoalloteichus alkalophilus
4	Actinobacillus capsulatus	17	Actinomadura Latina	31	Actinomyces canis
5	Actinomyces hyovaginalis	18	Actinopolymorpha	32	Actinopolymorpha alba
6	Actinopolyspora indiensis	19	Actinopolyspora salina	33	Adlercreutzia equolifaciens
7	Aequorivita sublithincola	20	Aerococcus viridans	34	Agrobacterium larrymoorei
8	Agrobacterium undicola	21	Agrobacterium viscosum	35	Agrococcus jejuensis
9	Alcanivorax indicus	22	Alkaliphilus transvaalensis	36	Allochromatium warmingii
10	Alteromonas alvinellae	23	Aminobacter ciceronei	37	Aminobacterium colombiense
11	Aminobacterium mobile	24	Amphritea atlantica	38	Aquicella siphonis
12	Arenimonas malthae	25	Arthrobacter soli	39	Aureispira maritima
		26	Azospira restricta	40	Azospirillum rugosum
	B				
41	Bacillus halmapalus	48	Bacillus butanolivorans	55	Bacillus djibelorensis
42	Bacteriovorax litoralis	49	Bacillus pseudomegaterium	56	Bacillus thermoamylovorans
43	Bacteroides vulgatus	50	Bacteroides helcogenes	57	Bacteroides nordii

44	Bifidobacterium boum	51	Bartonella weissi	58	Bifidobacterium asteroides
45	Blautia hansenii	52	Bifidobacterium cuniculi	59	Bifidobacterium gallinarum
46	Brenneria salicis	53	Brachybacterium	60	Brachybacterium arcticum
47	Brevundimonas terrae	54	Brevibacterium album	61	Brevibacterium mcbrellneri
	C				
62	Caldisphaera draconis	78	Caloramator fervidus	94	Caloramator proteoclasticus
63	Caminibacter profundus	79	Campylobacter hominis	95	Campylobacter hyointestinalis
64	Campylobacter peloridis	80	Campylobacter troglodytis	96	Candidatus Blochmannia pennsylvanicus
65	Candidatus Blochmannia vafer	81	Candidatus Endobugula	97	Candidatus Liberibacter
66	Candidatus Phytoplasma brasiliense	82	Candidatus Phytoplasma prunorum	98	Candidatus Rhabdochlamydia
67	Candidatus Scalindua brodae	83	Candidatus Tammella	99	Carnobacterium mobile
68	Cellulomonas denverensis	84	Cellvibrio ostraviensis	100	Chelatococcus daeguensis
69	Chitinophaga skermanii	85	Chlorobaculum parvum	101	Chondromyces apiculatus
70	Chondromyces robustus	86	Chryseobacterium formosense	102	Chryseobacterium soli
71	Citricoccus muralis	87	Citromicrobium bathyomarinum	103	Clostridium aurantibutyricum
72	Clostridium cavendishii	88	Clostridium cellulolyticum	104	Clostridium diolis
73	Clostridium gasigenes	89	Clostridium hveragerdense	105	Clostridium hydrogeniformans
74	Clostridium paradoxum	90	Clostridium subterminale	106	Clostridium thermobutyricum
75	Cohnella soli	91	Cohnella thermotolerans	107	Collinsella tanakaei
76	Comamonas kerstersii	92	Comamonas odontotermitis	108	Coprothermobacter platensis
77	Corynebacterium appendicis	93	Corynebacterium marinum	109	Corynebacterium riegelii
				110	Cryptosporangium arvum
	D				
111	Deefgea rivuli	130	Deferribacter thermophilus	150	Deinococcus gobiensis
112	Deinococcus piscis	131	Deinococcus radiophilus	151	Deinococcus soli
113	Delftia lacustris	132	Demequina salsinemoris	152	Dermacoccus barathri

114	Dermacoccus profundi	133	Desulfacinum subterraneum	153	Desulfofrigus oceanense
115	Desulfomicrobium baculatum	134	Desulfomicrobium escambiense	154	Desulfomicrobium macestii
116	Desulfomicrobium norvegicum	135	Desulfomicrobium orale	155	Desulfonatronovibrio
117	Desulfonatronovibrio hydrogenovorans	136	Desulfosarcina cetonica	156	Desulfosarcina ovata
118	Desulfosporosinus auripigmenti	137	Desulfotalea arctica	157	Desulfotignum phosphitoxidans
119	Desulfotomaculum acetoxidans	138	Desulfotomaculum putei	158	Desulfotomaculum reducens
120	Desulfotomaculum thermobenzoicum	139	Desulfovermiculus halophilus	159	Desulfovibrio aceae
121	Desulfovibrio burkinensis	140	Desulfovibrio capillatus	160	Desulfovibrio carbinolicus
122	Desulfovibrio cuneatus	141	Desulfovibrio ferrireducens	161	Desulfovibrio ferrophilus
123	Desulfovibrio frigidus	142	Desulfovibrio gracilis	162	Desulfovibrio idahonensis
124	Desulfovibrio inopinatus	143	Desulfovibrio lacusfryxellense	163	Desulfovibrio litoralis
125	Desulfovibrio longreachensis	144	Desulfovibrio marinus	164	Desulfovibrio oceani
126	Desulfovibrio oryzae	145	Desulfovibrio piger	165	Desulfovibrio salexigens
127	Desulfovibrio simplex	146	Desulfovibrio vietnamensis	166	Desulfurella propionica
128	Desulfuromusa succinoxidans	147	Dialister invisus	167	Dietzia cercidiphylli
129	Dietzia schimae	148	Dokdonella fugitiva	168	Dokdonella koreensis
		149	Dysgonomonas hofstadii	169	Dolichospermum curvum
	E				
170	Enterococcus rotate	172	Ectothiorhodospira imhoffii	174	Eikenella corrodens
171	Eubacterium callanderi	173	Enterococcus silesiacus	175	Erysipelothrix tonsillarum
	F				
176	Fervidobacterium pennivorans	178	Flammeovirga arenaria	180	Flavobacterium algicola
177	Flavobacterium anhuiense	179	Fructobacillus ficulneus		
	G				
181	Geobacter pelophilus	185	Geobacillus anatolicus	190	Geobacter chapelleii
182	Geobacter uraniireducens	186	Geobacter pickeringii	191	Geobacter toluenoxydans

183	Glaciecola punicea	187	Georgenia deserti	192	Giesbergeria voronezhensis
184	Gordonia defluvii	188	Glycomyces sambucus	193	Glycomyces tenuis
		189	Gordonia hirsuta	194	Gordonia polyisoprenivorans
	H				
195	Halanaerobium praevalens	201	Haloanella gallinarum	206	Halalkalicoccus tibetensis
196	Halomonas fontilapidosi	202	Halomonas gudaonensis	207	Halochromatium salexigens
197	Halomonas sinaiensis	203	Helicobacter rodentium	208	Halomonas hamiltonii
198	Herbaspirillum huttiense	204	Hydrocarboniphaga	209	Helicobacter salomonis
199	Hydrogenophilus thermoluteolus	205	Hyphomonas oceanitis	210	Hydrogenivirga okinawensis
200	Hyphomonas rosenbergii				
	J				
211	Janibacter limosus				
	K				
212	Kineosporia rhizophila	214	Kitasatospora melanogena	216	Kineosporia mikuniensis
213	Kribbella yunnanensis	215	Kutzneria kofuensis	217	Kribbella koreensis
	L				
218	Lampropedia hyalina	221	Lactobacillus antri	224	Lactobacillus taiwanensis
219	Leptospira fainei	222	Legionella tucsonensis	225	Lentzea waywayandensis
220	Lysobacter niabensis	223	Lutibacterium anuloederans	226	Lysobacter deserti
	M				
227	Maribacter goseongensis	236	Maricaulis parjimensis	245	Marinimicrobium koreense
228	Marinomonas blandensis	237	Marinomonas foliarum	246	Megamonas hypermegale
229	Megasphaera elsdenii	238	Methanobrevibacter acididurans	247	Microbacterium barkeri
230	Microbacterium pygmaeum	239	Microbulbifer okinawensis	248	Micrococcus thailandicus
231	Mogibacterium pumilum	240	Moorella glycerini	249	Moritella yayanosii
232	Mycetocola lacteus	241	Mycobacterium gilvum	250	Mycobacterium simulans

233	Mycoplasma alkalescens	242	Mycoplasma arginini	251	Mycoplasma auris
234	Mycoplasma canadense	243	Mycoplasma gypis	252	Mycoplasma haemocanis
235	Mycoplasma sualvi	244	Mycoplasma subdolum	253	Mycoplasma testudineum
	N				
254	Nautilia lithotrophica	259	Nesterenkonia halobia	264	Natronincola ferrireducens
255	Niastella koreensis	260	Nitrosococcus watsoni	265	Niabella soli
256	Nocardia polyresistens	261	Nocardioides islandensis	266	Nocardia alba
257	Nostoc flagelliforme	262	Novosphingobium acidiphilum	267	Nocardioides lentus
258	Novosphingobium taihuense	263	Novosphingobium yangbajingensis	268	Novosphingobium indicum
	O				
269	Oleomonas sagaranensis	271	Oceanisphaera laurenciae	272	Ochrobactrum pseudogrignonense
270	Olivibacter ginsengisoli				
	P				
273	Paenibacillus ruminocola	286	Paenibacillus donghaensis	298	Paenibacillus filicis
274	Pectinatus haikarae	287	Paenisporosarcina quisquiliarum	299	Pasteuria nishizawae
275	Pelagicoccus litoralis	288	Pedobacter agri	300	Pelagicoccus albus
276	Peptoniphilus tyrrelliae	289	Pelomonas saccharophila	301	Peptoniphilus asaccharolyticus
277	Phenylobacterium mobile	290	Peptostreptococcus anaerobius	302	Phenylobacterium immobile
278	Planctomyces limnophilus	291	Photobacterium halotolerans	303	Pimelobacter simplex
279	Prevotella brevis	292	Planomicrobium chinense	304	Porphyromonas bennonis
280	Prevotella disiens	293	Prevotella corporis	305	Prevotella denticola
281	Promicromonospora kroppenstedtii	294	Prevotella falsenii	306	Prevotella shahii
282	Prosthecochloris vibrioformis	295	Promicromonospora sukumoe	307	Propionibacterium avidum
283	Pseudochrobactrum saccharolyticum	296	Pseudidiomarina donghaiensis	308	Pseudoalteromonas gracilis
284	Psychrobacter proteolyticus	297	Pseudomonas syncyanea	309	Pseudonocardia sulfidoxydans
285	Pyramidobacter piscolens				

	R				
310	Rhodococcus baikonurensis	314	Rheinheimera chironomi	318	Rhizobium alamii
311	Rhodovulum robiginosum	315	Rhodococcus imtechensis	319	Rhodovulum imhoffii
312	Rivularia atra	316	Rickettsia limoniae	320	Rickettsia marmionii
313	Ruegeria pomeroyi	317	Rubrivivax gelatinosus	321	Ruegeria lacuscaerulensis
	S				
322	Sagittula stellate	335	Salegentibacter salegens	348	Salinivibrio siamensis
323	Salisaeta longa	336	Scardovia inopinata	349	Sedimentibacter saalensis
324	Shewanella upenei	337	Shimazuella kribbensis	350	Skermanella aerolata
325	Slackia faecicanis	338	Solirubrobacter soli	351	Sphaerochaeta globus
326	Sphingobium yanoikuyae	339	Sphingomonas abaci	352	Sporichthya polymorpha
327	Sporosarcina luteola	340	Sporotomaculum hydroxybenzoicum	353	Staphylococcus epidermidis
328	Staphylococcus felis	341	Staphylococcus fleurettii	354	Staphylococcus pseudolugdunensis
329	Streptacidiphilus jiangxiensis	342	Streptobacillus moniliformis	355	Streptococcus constellatus
330	Streptococcus downei	343	Streptococcus luteciae	356	Streptococcus macacae
331	Streptococcus massiliensis	344	Streptomyces minoensis	357	Streptomyces rajshahiensis
332	Streptomyces roseosporus	345	Streptomyces synnematoformans	358	Streptosporangium yunnanense
333	Stygiolobus azoricus	346	Succiniclasticum ruminis	359	Sulfurospirillum arcachonense
334	Syntrophomonas curvata	347	Syntrophomonas erecta	360	Syntrophomonas palmitatica
	T				
361	Tepidimonas thermarum	366	Tetragenococcus solitarius	371	Tepidimicrobium ferriphilum
362	Thermoanaerobacter sulfurophilus	367	Thermoanaerobacter thermocopriae	372	Thermoanaerobacter inferii
363	Thiocapsa litoralis	368	Thiomicrospira thermophila	373	Thiobacter subterraneus
364	Thiothrix nivea	369	Trabulsiella odontotermitis	374	Thioploca ingrica
365	Treponema berlinense	370	Treponema parvum	375	Treponema azotonutricium

				376	Treponema pectinovorum
	U				
377	Ureibacillus thermophiles				
	V				
378	Verminephrobacter eiseniae	380	Vibrio gazogenes	381	Vibrio mytili
379	Veillonella ratti				

TABLE 6: RELATIVE ABUNDANCE OF TOP 20 BACTERIAL SPECIES IN HEALTHY VERSUS GINGIVAL RECESSION SITES

S.No	SPECIES	HEALTH MEAN (%)	RECESSION MEAN (%)
1.	Fusobacterium naviforme	4.894	3.691
2.	Campylobacter gracilis	3.045	1.929
3.	Chryseobacterium taichungense	2.248	3.261
4.	**Zhouia amylolytica**	**1.981**	**0.409**
5.	**Capnocytophaga leadbetteri**	**1.464**	**0.307**
6.	Campylobacter showae	1.325	1.762
7.	**Fusobacterium periodonticum**	**1.207**	**0.259**
8.	Fusobacterium nucleatum	1.062	0.809
9.	Streptococcus tigurinus	0.851	0.409
10.	Mannheimia caviae	0.793	0.107
11.	Porphyromonas gingivalis	0.727	2.188
12.	Spingobacterium shayense	0.673	0.050
13.	**Fusobacterium gonidiformins**	**0.612**	**0.181**
14.	**Capnocytophaga ochracea**	**0.609**	**0.223**
15.	Porphyromonas catoniae	0.568	0.154
16.	Selenomonas artemidis	0.561	0.396
17.	Neisseria mucosa	0.517	0.346
18.	**Capnocytophaga gingivalis**	**0.492**	**0.039**
19.	Selenomonas infelix	0.485	1.134
20.	Neisseria lactamica	0.446	0.243

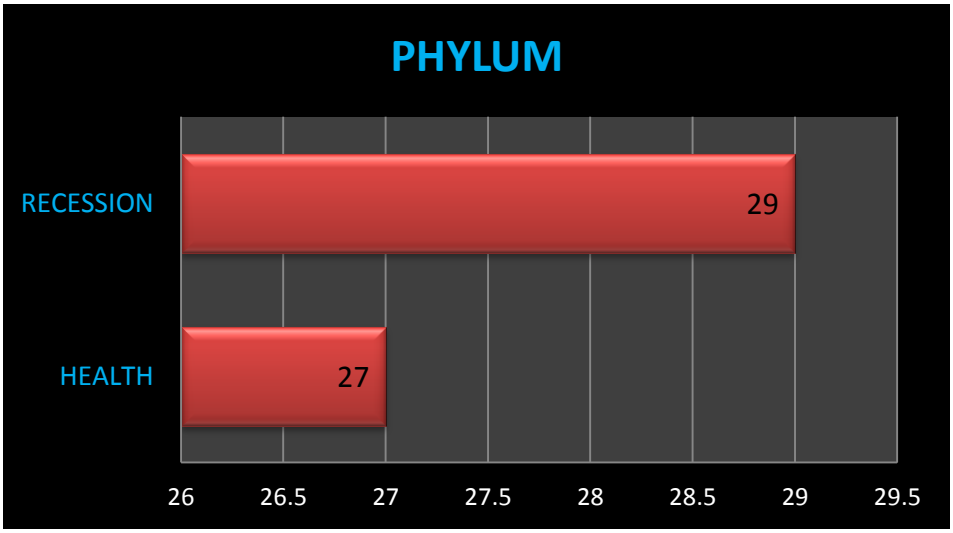
Statistically significant species ($p<0.05$) between healthy VS Gingival recession Sites

TABLE 7: RELATIVE ABUNDANCE OF TOP 20 BACTERIAL SPECIES IN GINGIVAL RECESSION VERSUS HEALTHY SITES

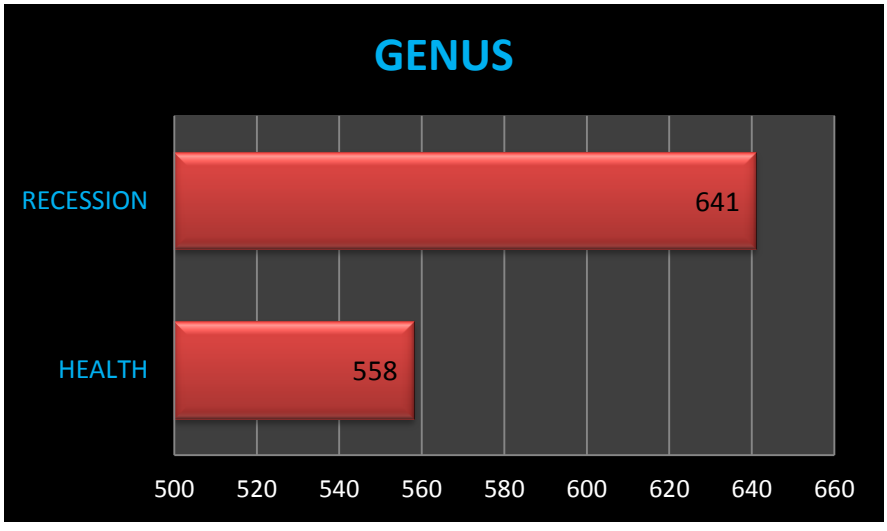
S.No	SPECIES	RECESSION MEAN (%)	HEALTH MEAN (%)
1.	Fusobacterium naviforme	3.691	4.894
2.	Chryseobacterium taichungense	3.261	2.248
3.	Porphyromonas gingivalis	1.966	0.727
4.	Campylobacter gracilis	1.926	3.045
5.	Campylobacter showae	1.763	1.325
6.	Treponema succinifaciens	1.477	0.001
7.	Megasphaera geminatus	1.221	0.072
8.	Selenomonas infelix	1.134	0.485
9.	Snowella rosea	1.122	0.084
10.	Pectinatus cerevisiiphilus	0.838	0.161
11.	Fusobacterium nucleatum	0.809	1.062
12.	Treponema parvum	0.635	0.01
13.	Comamonas koreensis	0.589	0.296
14.	Streptococcus tigurinus	0.409	0.851
15.	**Zhouia amylolytica**	**0.409**	**1.981**
16.	Tannerella forsythia	0.404	0.385
17.	Selenomonas artemidis	0.396	0.559
18.	Megasphaera hominis	0.389	0.022
19.	Corynebacterium matruchotii	0.382	0.141
20.	Neisseria mucosa	0.378	0.517

Statistically significant species ($p<0.05$) between Gingival recession VS healthy sites

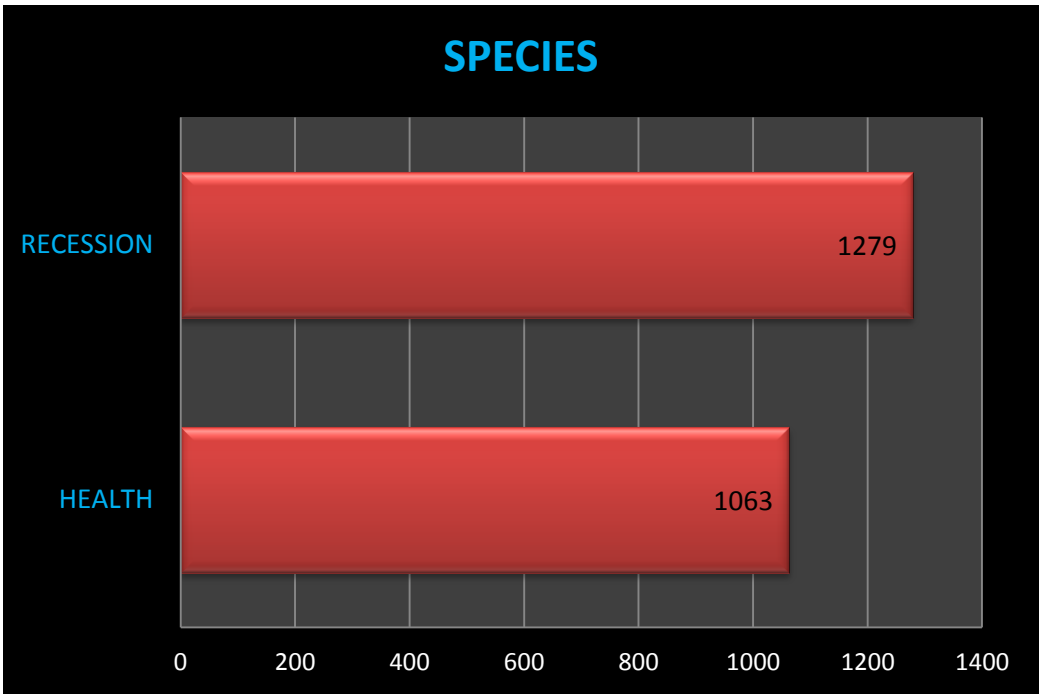
GRAPH 1-A: BAR GRAPH DEMONSTRATING DISTRIBUTION OF BACTERIAL PHYLUM IN HEALTH AND GINGIVAL RECESSION



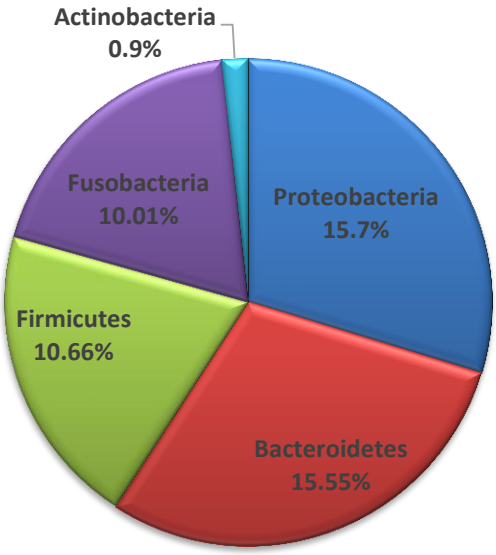
GRAPH 1-B: BAR GRAPH DEMONSTRATING DISTRIBUTION OF BACTERIAL GENUS IN HEALTH AND GINGIVAL RECESSION



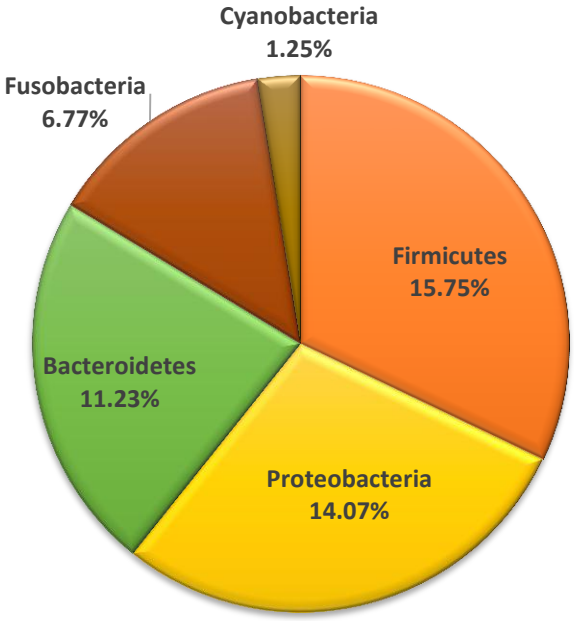
GRAPH 1-C: BAR GRAPH DEMONSTRATING DISTRIBUTION OF BACTERIAL SPECIES IN HEALTH AND GINGIVAL RECESSION



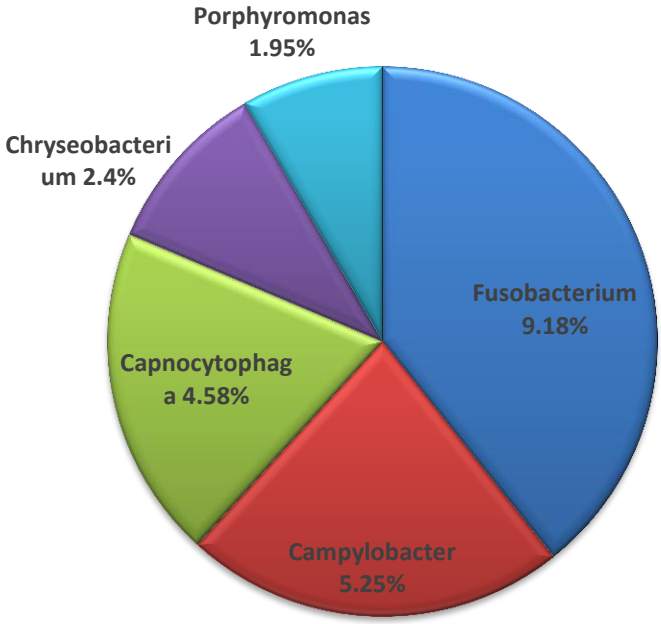
GRAPH 2-A: TOP 5 PHYLA IN HEALTH



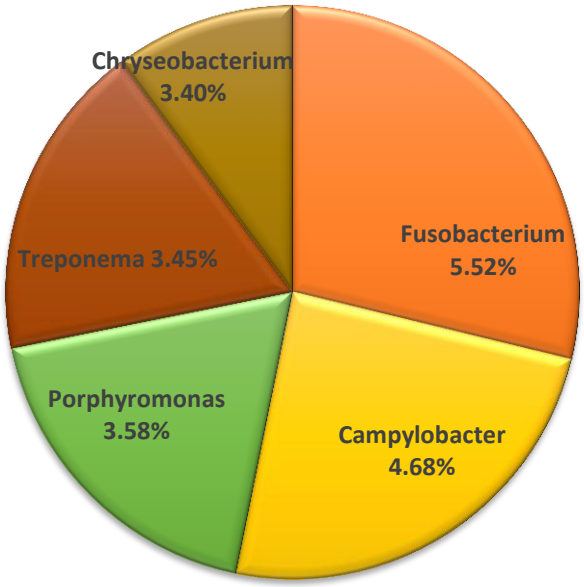
GRAPH 2-B: TOP 5 PHYLA IN GINGIVAL RECESSION



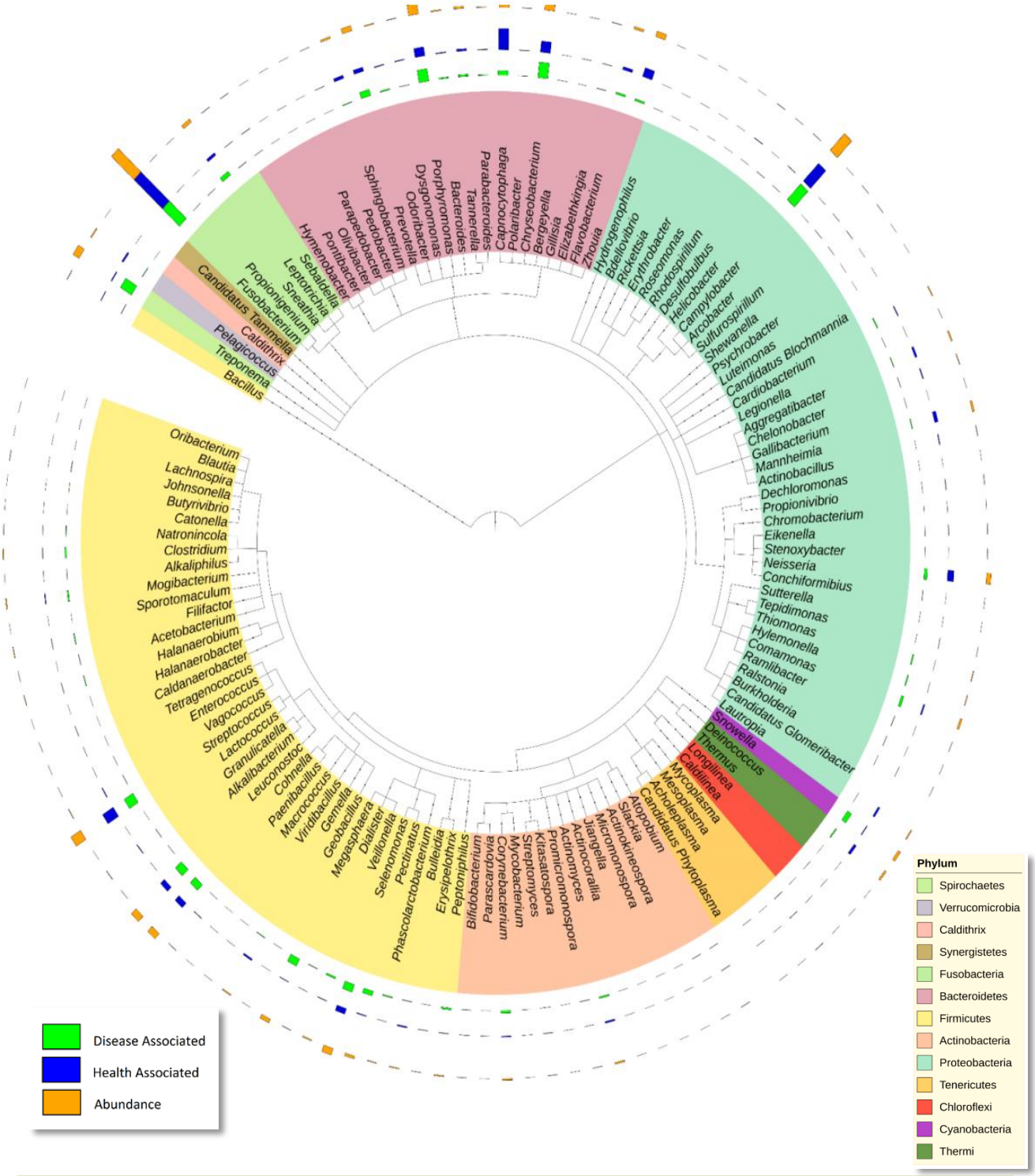
GRAPH 3-A: TOP 5 GENERA IN HEALTH



GRAPH 3-B: TOP 5 GENERA IN GINGIVAL RECESSION

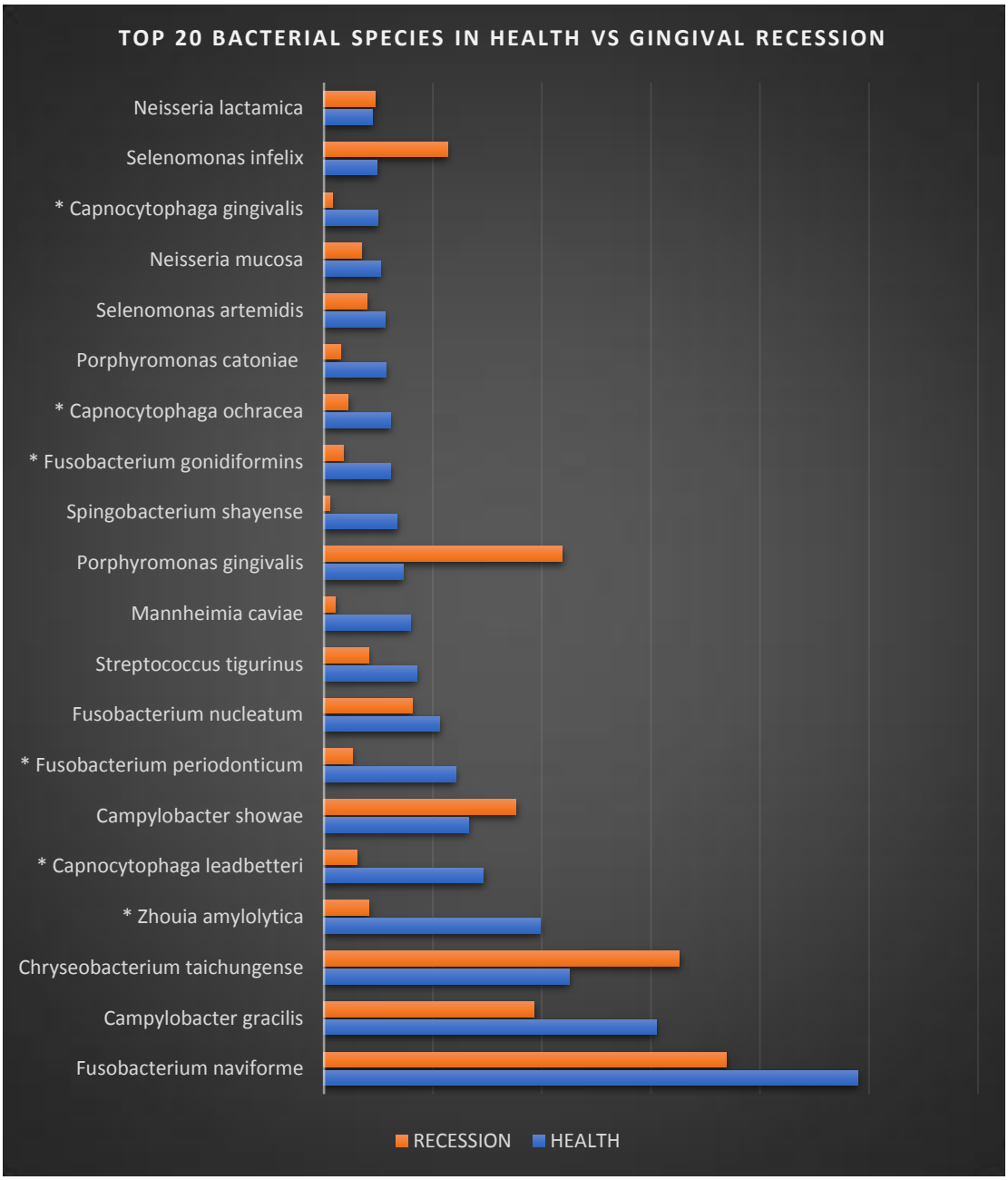


GRAPH 3-C: PHYLOGENETIC TREE AT GENUS LEVEL



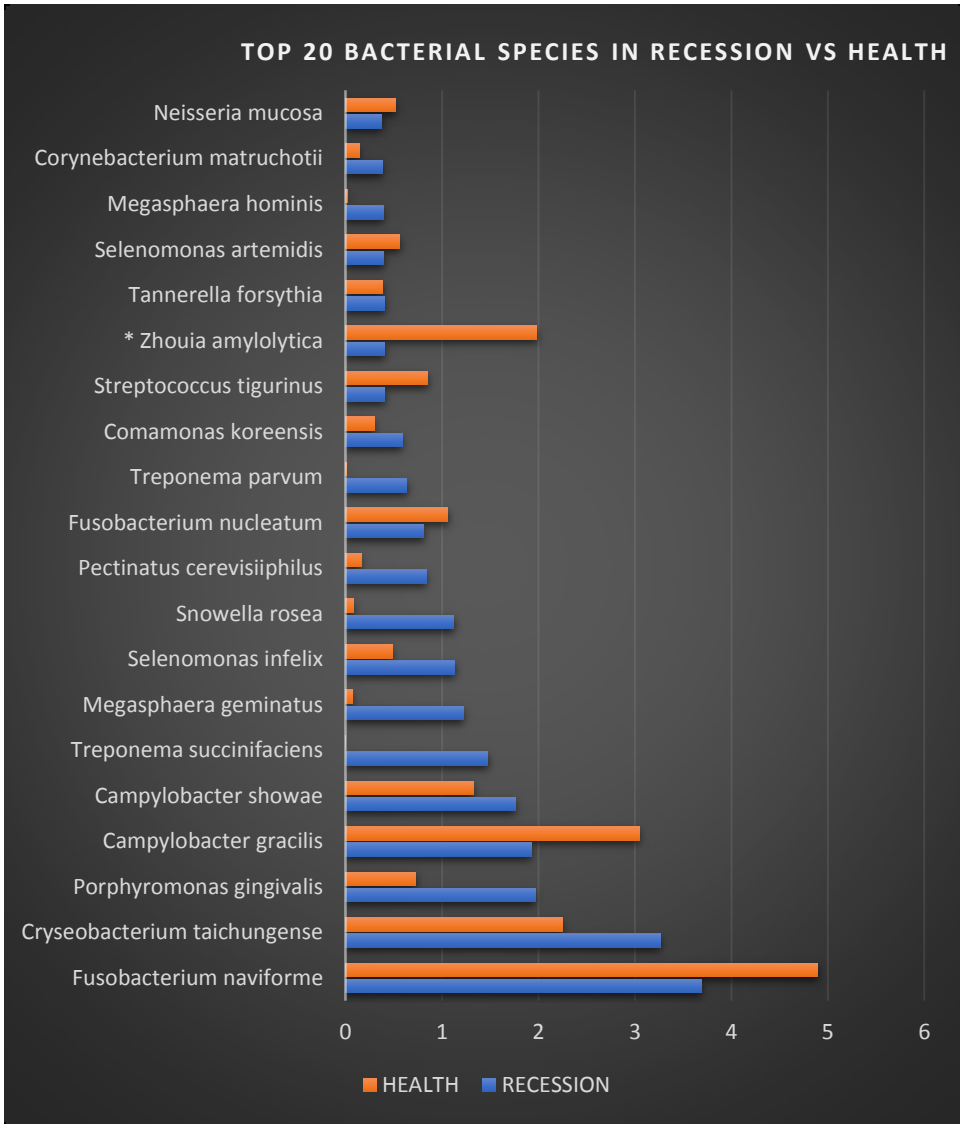
Circular maximum likelihood phylogenetic tree at the genus level. The tree was constructed with phyloT software and displayed using iTOL **(Letunic and Bork, 2011).** The bars in the outer band (orange) represent the relative abundance of bacterial genus in the healthy (blue) and the periodontal disease (green) groups.

GRAPH 4: BAR GRAPH SHOWING PERCENTAGE OF ABUNDANCE OF TOP 20 BACTERIAL SPECIES IN HEALTHY AND GINGIVAL RECESSION SITES



***indicates statistical significance (p value <0.05)**

GRAPH 5: BAR GRAPH DEMONSTRATING ABUNDANCE PERCENTAGE OF TOP 20 SPECIES IN GINGIVAL RECESSION AND HEALTHY SITES



***indicates statistical significance (p value <0.05)**

Discussion

DISCUSSION

Periodontitis is a chronic inflammatory disease of polymicrobial origin characterized by tissue destruction and eventually loss of supporting periodontium. Various hypotheses have been proposed to substantiate its microbial etiology such as nonspecific plaque hypothesis,[13,76] specific plaque hypothesis,[56] ecologic plaque hypothesis[72] and keystone pathogen hypothesis.[35] **Hajishengallis** proposed PSD model in 2012 which has been accepted by several researchers in this field. PSD model states that synergistic events between multiple microbial communities and dysbiosis (perturbations in structure and composition of bacterial communities) in a susceptible host results in periodontitis.[35]

Microbes live synergistically within human body and contribute to maintenance of oral health and homeostasis. Dysbiotic changes in subgingival microbiome and community structures can contribute to pathogenesis of periodontal diseases.[12] There are several ecological niches within the oral cavity that may have distinct microbiome of its own, and these may vary dynamically over time due to daily activities such as brushing teeth, drinking juices, smoking and eating. Due to these variations it is difficult to associate causative pathogens with periodontal diseases from a small group of subjects.[136]

Turnbaugh et al[118] described human microbiome to be classified into a core microbiome and a variable microbiome. Core microbiome comprises of the predominant species that exist under healthy conditions at different sites of the body, and it is shared by all individuals.[109,118,132] Variable microbiome is one that has evolved in response to unique lifestyle, phenotypic and genotypic determinants; it is exclusive for an individual.

Several studies on subgingival microbiome have documented data regarding periodontal pocket[54,79,87,105] but there is paucity in literature regarding subgingival microbiome in gingival recession sites. The ecological niche of gingival recession sites is found to be different from that of periodontal pocket sites and healthy sites. To the best of our knowledge, no previous studies have reported the differences at levels of phylum, genus and species between health and gingival recession sites with gingival inflammation.

Among the various microbial diagnostic techniques like PCR and immunodiagnostics, advances in DNA sequencing and bioinformatics technologies have made possible two orders of magnitude higher resolution of bacterial community composition.[32] Application of molecular biology based diagnostic techniques to identify and clarify bacterial taxonomy has been on rise over the last decade. rRNA sequences have been used for accurate and rapid identification of known species of bacteria without any need for strain cultivation. Along with nucleotide amplification technology several unknown species could be differentiated, quantified and identified.[112] Owing to various

merits as credited by earlier researchers[32,42,54] NGS technology, an open-ended technique known for its high-throughput genomic analysis approach and ability to quantify abundance of bacterial species, has been applied in this study for sequencing microbiome of subgingival plaque samples from gingival recession patients and periodontally healthy subjects. Illumina sequencing, an advent of NGS technology[46,132] has been used in this study as it provides more sequences per run, analyzes larger sample size, better assessment of microbiome diversity, inclusion of more bar-coded time points and samples, generates and sequences short 16S rRNA amplicons to determine even low abundance taxa.

V3-V4 region of 16S rRNA was used in this study because though V4 region provides full overlap of two reads and reduces noise in sequencing data thus preventing OTU inflation, there is only less information contained in V4 region owing to its length (~255 base pairs). A longer fragment such as V3 which spans multiple hypervariable regions is most suitable for distinguishing all bacterial species to genus level.[121]

Subjects were periodontally evaluated and allocated into two groups as healthy controls (four subjects) and localized chronic periodontitis patients (four subjects) with sites exhibiting gingival recession associated with local factors and gingival inflammation. Sterile Gracey curettes were used to collect subgingival plaque samples owing to their reliability as observed in earlier literature.[54] Alternate techniques such as paper points allowed only passive

translocation of plaque material and fluid into sampling devices which is likely to have represented only outer biofilm microorganisms, thus undersampling initial colonizers present in the inner biofilm mass attached to root surface.[32] In the experimental workflow, all reactions were carried out with water and plastic materials guaranteed as DNA-free to avoid contamination.

Vast diversity of subgingival microflora and inter-individual variations among all samples were observed in our study. A total of 27 phyla, 558 genera and 1063 species in healthy sites; and 29 phyla, 641 genera and 1279 species in gingival recession sites were observed and reported in our study. Although no significant difference was observed in between groups at phylum level, a definite increase in number of bacteria was identified at genus and species levels in gingival recession sites when compared to health.

900 species were observed to be commonly present in both health and recession sites; 161 species were unique to health and 381 species unique to disease. This numerical data was higher than earlier reports by **Griffen et al**[32] which could be due to application of Illumina technique in our study that could have aided in identifying larger number of species in health and disease samples.

Several studies have reported differences in microbiome at phylum level[32,54,63,117] and this database (Silva) was used in this study. The results of our study too fall in line with these earlier studies, with the top five phyla in

health being Firmicutes (15.75%), Proteobacteria (14.07%), Bacteroides (11.23%), Fusobacteria (6.77%) and Cyanobacteria (1.25%). The finding that Firmicutes were the most predominant phyla in healthy sites is not surprising, considering that the Gram-positive cocci comprising early colonizers such as Streptococcus, Selenomonas, Pectinatus and Cohnella belong to this phylum. In comparison with results from earlier studies, our study also reports a higher abundance of Proteobacteria and Fusobacteria. Proteobacteria phylum comprises of species such as Campylobacter, Hemophilus, Mannheimia and Desulfobulbus and Fusobacteria phylum comprises genera Fusobacterium and Leptotriciae; all of which are known to be early colonisers.[112]

The top 5 phyla in gingival recession sites are Proteobacteria (15.69%), Bacteroidetes (15.55%), Firmicutes (10.66%), Fusobacteria (10.01%) and Actinobacteria (0.9%). In a study conducted by **Wang et al**[124] top four phyla present in disease samples were reported as Bacteriodetes, Actinobacteria, Proteobacteria and Firmicutes, which is in line with results of our study. In recession sites, Proteobacteria was observed to be the predominant phylum in our study. It is one of the largest bacterial phyla and contains most of the Gram negative bacteria known to be periopathogens such as Actinobacter and Hemophilus (gamma division), *Eikenella corrodens* (beta division) and Campylobacter (epsilon division).[112]

Phylum Bacteriodetes was the second most abundant phylum present in gingival recession sites (15.55%) and was observed to be significantly higher than in healthy sites (11.23%) which were in concordance with reports

by **Griffen et al**.[32] This phylum is predominantly represented by Porphyromonas genus (bacteroides subgroup) of which *Porphyromonas gingivalis* is a classic red complex microorganism.[105]

The abundance of Firmicutes in recession sites can be attributed to the complexity of subgingival biofilm which allows survival of Gram positive microbes in a supposedly hostile anaerobic niche which was reported earlier by **Abusleme et al**.[3]

Phyla such as Spirochetes and Thermi contribute to minor proportions of subgingival microbiome in line with studies by **Hong et al**[42] and **Kumar et al**.[54] When bacteria were analyzed at phyla level, TM7 was not identified in our study. Although members of this phylum are yet to be cultured or classified at species level, reports indicate that members in this phylum may contribute to periodontal disease progression.[3,32,63,68]

At genus level, Fusobacterium was the predominant genus among healthy sites (9.1%) and gingival recession sites (5.52%). This was in line with earlier studies done by **Keijser BJ et al**[46], **Liu B et al**[63], **Griffen AL et al**[32], **Shi B et al**[102]. Species from Fusobacterium genus play a major role as bridging organisms in establishing periodontitis, and as a link between early and late colonizers within plaque biofilm.

Porphyromonas was the third most prominent genus identified in gingival sites. It comprises of anaerobic Gram-negative non motile cocci which have been established as species contributing to periodontal

pathogenesis.[32,46,63,102] This finding is in correlation to the increased prevalence of phylum Bacteroidetes in our study as reported before.

Treponema was identified predominantly from gingival recession sites, and is the fourth most predominant genus to be reported (3.4%) in the recession group. The mean abundance of Treponema genus was 1.7% in health and 3.7% in disease with greater abundance noted in disease (data not tabulated). Ten species of Treponema have been cultivated from the oral cavity and in different pocket depths.[6] Treponema genus consists of obligate anaerobes and microaerophiles. It represents a genus of commonly found oral bacteria that have been implicated in periodontal etiopathogenesis,[3,32,63] thus establishing their virulence as a periopathogen.

At species level, a distinct health-associated microbiome and disease-associated microbiome was identified. Among the 1063 species identified in health and 1279 species identified in disease, 161 and 381 species were uniquely present in health and disease respectively. On the basis of relative abundance profiles of the bacterial genera, we found two distinct groups of bacterial organisms—disease-associated and health-associated organisms. The subgingival microbiome was dominated by anaerobes in the diseased state and by facultatively anaerobic or aerobic organisms in healthy sites. Out of these, the top 20 most abundant species and those commonly associated with periodontal health and disease have been taken into consideration for discussion in this study.

Among the species belonging to Fusobacterium genus, *Fusobacterium naviforme* was identified to be the predominant bacteria in health (4.89%) and in recession sites (3.69%); *Fusobacterium periodonticum, Fusobacterium nucleatum and Fusobacteirum gonidiformins* were at the seventh, eighth and thirteenth positions respectively among the top 20 species in health. *F.periodonticum* and *F.gonidiformins* showed a statistically significant increase among health sites than recession sites. It is a well-established fact that Fusobacterium species play a major role as bridging organism in establishing periodontitis. *Fusobacterium nucleatum* is important for co-aggregation between early and late colonizers, thereby helping in subgingival biofilm organization; this organism may be used as marker for transition from gingivitis to periodontitis and for further disease progression.[3,32] The observation that Fusobacterium was the predominant genus identified in our study adds value to this fact too.

F.naviforme and *Megasphaera geminatus* were reported to be elevated in subgingival microbiome of periodontally healthy subjects who were smokers along with several other pathogenic species.[73] The anaerobic environment observed subgingivally in a clinically healthy smoker is similar to the one in deep pockets, thus supporting the shift in microbial community from aerobic commensal-rich niche to anaerobic, highly diverse and pathogen-rich niche.

Campylobacter species comprising C. gracilis and C. showae showed greater preponderance in health when compared to disease as proven by earlier

studies[2] which have shown that these species were associated with bacterial profiles of subgingival plaque in healthy subjects. It is a genus of Gram-negative bacteria belonging to phylum Proteobacteria, and this finding correlates to our earlier finding that Prteobacteria was the predominant phylum.

Porphyromonas species especially P.gingivalis and P. catoniae occupied 11th and 15th position in top 20 species of health. This genus mostly consists of non-motile, Gram-negative, anaerobic pathogenic bacteria which play a pivotal role in pathogenesis of periodontitis.[112]

Treponema as a genus was featured in top 5 genera only in recession sites. Its species T.succinifaciens and T.parvum are in 6^{th} and 12^{th} positions respectively.

Species belonging to Capnocytophaga genus were found to be associated with subgingival healthy and recession sites; C. leadbetteri, C.ochracea and C.gingivalis were abundant in 5^{th}, 14^{th} and 18^{th} positions respectively in health.[2] These species were associated with bacterial profiles of tooth surface in healthy subjects which could have become a part of the subgingival plaque over a period of time. These species showed statistically significant increases in healthy sites when compared to recession sites.

The red complex bacteria proposed by Socransky and Haffajee, namely *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* were detected in both healthy and recession groups with no significant differences in abundance. Among the top 20 species listed in recession sites,

only *Porphyromonas gingivalis* and *Tannerella forsythia* were found at species level. Treponema was identified in abundance only at genus level. *P.gingivalis* was observed to be the third most abundant species in recession sites, and although it was observed in health sites as well its mean abundance was higher in recession sites than health; *Tannerella forsythia* was also observed to be marginally increased in disease sites compared to health sites, thus establishing their role in tissue destruction and progression of periodontal disease.[88,135] It was a surprising coincidence that in our study T.forsythia occupied 16th position in relative abundance in diseased sites, a finding that was exactly similar to a study reported by **Griffen et al.**[32]

P.gingivalis has been proposed to play a central role in progression of human periodontitis and thus is classified as a "keystone pathogen" which influences composition of oral microbiome even when present at low levels.[19]

T.denticola, a potent red complex periopathogen, was identified only among three disease samples and not in health. Among the disease samples the abundance was as low as 0.3%. These results are not in agreement with **Socransky and Haffajee,**[108] **Ximenez-Fyvie et al**[129] who have described red complex bacteria as climax colonizers thought to be most associated with periodontal disease. These results are however in agreement with studies by **Kumar et al**[54] and **Griffen et al**[32] who have shown that there are no significant difference in presence of red complex bacteria in health and disease when the microbiome as a whole was studied.

The Streptococci species mainly *Streptococcus sanguinis, Streptococcus gordonii, Streptococcus mitis, Streptococcus intermedius,* and *Streptococcus oralis* are also part of early colonizers that have been described by **Sockransky and Haffajee**.[105] These bacteria are gram positive, aerobic, carbohydrate utilizing bacteria which are able to colonize on the acquired pellicle on tooth surfaces thus initiating plaque formation. These bacteria utilize available resources, create a bacterial succession through an ecological shift as described by Marsh and contribute to formation of late colonizers. The results of our study suggest that genus Streptococcus is found at a mean abundance of 2.7% in recession samples and 3.5% in health samples. Though there was no significant difference in abundance of Streptococci between health and disease, the fractionally higher abundance in health is in agreement with previous literature in relation to subgingival microbiome.[32,46,63,102]

As reported by earlier studies[62,65] a diverse and rich microbial community was detected in periodontitis than health. Even among chronic periodontitis samples, health-associated species were found to be accounted in a small fraction of the total community, establishing the fact that subgingival biofilm represents a more stable and healthy ecosystem. A varied ecosystem would thus undermine host defenses which would otherwise favor a non-pathogenic community to prevail. It also must be noted that since most health-associated species are also found in chronic periodontitis though in lesser

fraction, introducing healthy species using probiotics may not be effectual in altering the course of disease towards health.

Previous results from a study done in our department (unpublished data) have documented the subgingival microbiome associated with periodontal pockets in an Indian population. The microbiome in gingival recession was by and large closer to that associated with periodontal pocket than with health. However, subtle changes in the microbiome in recession sites have been established with certain species featuring uniquely in gingival recession sites and not found in pocket sites. These may be responsible for differences in etiopathogenic mechanisms between gingival recession and periodontal pocket. Although chronic periodontitis subjects included in our study exhibited gingival recession owing to chronic gingival inflammation, recession still as an ecosystem is not entirely soft tissue related unlike periodontal pocket which has several tissue invasive micro-organisms deep within epithelium and connective tissue. After the ensuing inflammation and loss of attachment owing to marginal tissue migration, recession sites require tooth adhering microbes to progress further. These microbial pathogens like *C. gracilis, F.nucleatum, N. mucosa* are associated with bacterial profile on tooth surface associated with health. They utilize glycoproteins on tooth surface for their nutritional requirements and sustenance.

Our results support the hypothesis that subgingival biofilm as a whole and dysbiosis in particular may contribute more to pathogenesis of periodontal disease rather than individual bacteria. There was a distinct bacterial species in

disease associated microbiome when compared to health. The traditional periodontal pathogens (red complex bacteria and newer ones Filifactor, Dialister) seem to have a limited role in disease pathogenesis. Novel bacteria seem to be closely associated with recession but further studies need to be done to ascertain their etiopathogenic role. The results indicate that dietary patterns and lifestyle habits could have contributed to a microbial profile that has not been reported in previous literature. Greater carbohydrate content in the diet could have allowed presence of normal sacchrolytic bacteria and a neutral to mildly alkaline pH environment could have favoured growth of novel subgingival species such *Zhouia amylolytica, Chryseobacterium taichungense, Alkaliphilus crotonatoxidans* and *Pectinatus cerevisiiphilus*.

Bacteria may directly act on periodontal tissues as pathogens or indirectly stimulate damaging host inflammatory response. Traditional studies involved a 'reductionist' approach to analyze complex subgingival microbial communities thus regarding red complex microbes as the major putative periopathogens for a very long time. However, recent studies which employed advanced sequencing technologies have exhibited that instead of a single or group of microorganisms playing a role as pathogens; a diverse community of bacteria interacts synergistically resulting in pathogenesis. Metagenomic technologies have established the concept that each and every individual member of this diverse community could be involved in disease occurrence. Thus a species could be low in abundance but still attribute to the community as a critical species in disease progression.

Limitations of the study:

Smaller sample size and inability to obtain exact quantification of bacterial species using NGS technology is a limitation to our study. The vast diversity of subgingival microbiome identified and overlap of species among health and disease reaffirm that targeted antimicrobial approach against individual or group of bacteria may not be ideal for management of periodontal disease, thus warranting focus towards a more personalized periodontal medicine.

Summary and Conclusion

SUMMARY AND CONCLUSION

This study characterized subgingival microbiome in gingival recession sites and compared it with periodontally healthy sites. Eight subgingival samples including four periodontally healthy and four localized chronic periodontitis samples were collected and microbiome characterization was done with NGS technology using Illumina sequencing.

A wide array of microbes were identified in health and disease belonging to a complex community structure comprising 27 phyla, 558 genera and 1063 species identified among healthy sites and 29 phyla, 641 genera and 1279 species among gingival recession sites. Among the species characterized in our study, 161 were identified to be unique to health and 381 were unique to disease.

Although distinct health and disease associated microbiome were identified, there was low abundance of disease-associated species in health and health-associated species in disease. Traditional periodontopathogenic bacteria such as *Porphyromonas gingivalis, Tannerella Forsythia, Treponema denticola,* and newer periodontopathogens such as *Filifactor alocis, Dialister invisus* showed no significant difference in abundance in health and disease. There were no previous reports available on microbiome in gingival recession sites for comparison with our study results in particular. Further studies need to be done to identify the role of these bacteria in periodontal health and disease.

Bibliography

BIBLIOGRAPHY

1. **Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J.** The placenta harbors a unique microbiome. Science translational medicine. 2014;6(237):237.
2. **Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE.** Defining the normal bacterial flora of the oral cavity. Journal of clinical microbiology. 2005;43(11):5721-32.
3. **Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, Gamonal J, Diaz PI.** The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. The ISME journal. 2013;7(5):1016-25.
4. **Adessi C, Matton G, Ayala G, Turcatti G, Mermod JJ, Mayer P, Kawashima E.** Solid phase DNA amplification: characterization of primer attachment and amplification mechanisms. Nucleic Acids Res 2000;28:e87.
5. **Albandar JM, Kingman A.** Gingival recession, gingival bleeding, and dental calculus in adults 30 years of age and older in the United States, 1988–1994. J Periodontol 1999;70:30–43.
6. **Asai Y, Jinno T, Igarashi H, Ohyama Y, Ogawa T**. Detection and quantification of oral treponemes in subgingival plaque by real-time PCR. Journal of clinical microbiology. 2002;40(9):3334-40.
7. **Attia TH, Saeed MA.** Next Generation Sequencing Technologies: A Short Review. Next Generat Sequenc & Applic S. 2016;1:2.

8. **Avila-Campos MJ**. PCR detection of four periodontopathogens from subgingival clinical samples. Braz J Microbiol 2003;34:81-84.
9. **Baker D.L., & Seymour, D.J**. The possible pathogenesis of gingival recession. A histological study of induced recession in the rat. J Clin Periodontol 1976;3:208-219.
10. **Bearfield C, Davenport ES, Sivapathasundaram V, Allaker RP**. Possible association between amniotic fluid micro-organism infection and microflora in the mouth. BJOG: An International Journal of Obstetrics & Gynaecology. 2002;109:527-33.
11. **Bentley DR**. Whole-genome resequencing. Curr Opin Genet Dev. 2006;16:545-52.
12. **Berezow AB, Darveau RP**. Microbial shift and periodontitis. Periodontol 2000 2011;55:36–47.
13. **Black GV.** The Formation of Poisons by Micro-organisms: A Biological Study of the Germ Theory of Disease. P. Blakiston, son & Company; 1884.
14. **Blainey P.** The future is now: single-cell genomics of bacteria and archaea. FEMS Microbiol Rev,2013.
15. **Chambrone LA, Chambrone L.** Subepithelial connective tissue grafts in the treatment of multiple recession-type defects. J Periodontol 2006; 77:909–16.

16. **Chen H, Jiang W**. Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. Frontiers in microbiology. 2014;5.
17. **Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE.** The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database. 2010;2010.
18. **Costello EK, Carlisle EM, Bik EM, Morowitz MJ, Relman DA.** Microbiome assembly across multiple body sites in low-birthweight infants. MBio. 2013;4:e00782-13.
19. **Darveau RP.** The oral microbial consortium's interaction with the periodontal innate defense system. DNA and cell biology. 2009;28:389-95.
20. **Dethlefsen L, McFall-Ngai M, Relman DA.** An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature. 2007 18;449:811-8.
21. **Do T, Devine D, Marsh PD.** Oral biofilms: molecular analysis, challenges, and future prospects in dental diagnostics. Clinical, cosmetic and investigational dentistry. 2013;5:11.
22. **Dohm JC, Lottaz C, Borodina T, Himmelbauer H**. Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. Nucleic Acids Res 2008;36: e105.

23. **Donkor ES.** Sequencing of bacterial genomes: principles and insights into pathogenesis and development of antibiotics. Genes. 2013 14;4:556-72.

24. **Dorfman HS, Kennedy JE, Bird WC**. Longitudinal evaluation of free autogenous gingival grafts. J Clin Periodontol 1980;7:316–324.

25. **Dzink JL, Socransky SS, Haffajee AD.** The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. Journal of clinical periodontology. 1988;15:316-23.

26. **Dzink JL, Tanner AC, Haffajee AD, Socransky SS.** Gram negative species associated with active destructive periodontal lesions. Journal of clinical periodontology. 1985;12:648-59.

27. **Feres, M.** Change in subgingival microbial profiles in adult periodontitis subjects receiving either systemically-administered amoxicillin or metronidazole. J Clin Periodontol, 2001;28: p.597-609.

28. **Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, et al.** Whole-genome random sequencing and assembly of Haemophilus influenzae. Science 1995;269:496-512

29. **Freedman AL, Salkin LM, Stein MD, Green K.** A 10-year longitudinal study of untreated mucogingival defects. J Periodontol 1992;63:71–72.

30. **Ge X, Rodriguez R, Gunsolley J, Xu P.** Oral microbiome of deep and shallow dental pockets in chronic periodontitis. PloS one. 2013;8:e65520.
31. **Griffen A, Beall C, Firestone N, et al**. CORE: a phylogenetically-curated 16S rDNA database of the core oral microbiome. PLoS One. 2011;6:e19051.
32. **Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, Podar M, Leys EJ.** Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. The ISME journal. 2012;6:1176-85.
33. **Gurenlian JR.** The role of dental plaque biofilm in oral health. American Dental Hygienists Association. 2007;81:116
34. **Haffajee AD, Socransky SS**. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994;5:78-111.
35. **Hajishengallis G, Darveau RP, Curtis MA.** The keystone-pathogen hypothesis. Nature Reviews Microbiology. 2012;10:717-25.
36. **Hajishengallis G, Lamont RJ**. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Molecular oral microbiology. 2012;27:409-19.
37. **Hamady M, Knight R**. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome research. 2009;19:1141-52.

38. **Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, Beeson KY, Schork NJ, Murray SS, Topol EJ, Levy S, Frazer KA**. Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol 2009;10:R32.
39. **Hasan A, Palmer RM**. A clinical guide to periodontology: Pathology of periodontal disease. British dental journal. 2014;216:457-61.
40. **He X, McLean JS, Guo L, Lux R, Shi W.** The social structure of microbial community involved in colonization resistance. The ISME journal. 201;8:564-74.
41. **Hiyari S, Bennett KM.** Dental diagnostics: molecular analysis of oral biofilms. American Dental Hygienists Association. 2011;85:256-63.
42. **Hong BY, Araujo MV, Strausbaugh LD, Terzi E, Ioannidou E, Diaz PI.** Microbiome profiles in periodontitis in relation to host and disease characteristics. PloS one. 2015;10:e0127077.
43. **Hujoel PP, Cunha- Cruz J, Selipsky H, Saver BG**. Abnormal pocket depth and gingival recession as distinct phenotypes. Periodontology 2000. 2005;39:22-9.
44. **Jarosz LM, Ovchinnikova ES, Meijler MM, Krom BP.** Microbial spy games and host response: roles of a Pseudomonas aeruginosa small molecule in communication with other species. PLoS pathogens. 2011;7:e1002312.

45. **Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, Fernández L, Rodríguez JM**. Is meconium from healthy newborns actually sterile?. Research in microbiology. 2008;159:187-93.

46. **Keijser BJ, Zaura E, Huse SM, Van Der Vossen JM, Schuren FH, Montijn RC, Ten Cate JM, Crielaard W.** Pyrosequencing analysis of the oral microflora of healthy adults. Journal of dental research. 2008;87:1016-20.

47. **Kesic L, Milasin J, Igic M, Obradovic R.** Microbial etiology of periodontal disease–mini review. Med Biol. 2008;15:1-6.

48. **Keyes PH, Jordan HV**. Periodontal lesions in the Syrian hamster—III: Findings related to an infectious and transmissible component. Archives of oral biology. 1964;9:377-400.

49. **Kim J, Amar S**. Periodontal disease and systemic conditions: a bidirectional relationship. Odontology. 2006;94:10-21

50. **Kisch J, Badersten A, Egelberg J.** Longitudinal observation of "unattached", mobile gingival areas. J Clin Periodontol 1986;13:131–134.

51. **Kolenbrander PE**. Oral microbial communities: biofilms, interactions, and genetic systems. Annual Reviews in Microbiology. 2000;54:413-37.

52. **Korbel JO, Urban AE, Affourtit JP, Godwin B, Grubert F, Simons JF, Kim PM, Palejev D, Carriero NJ, Du L, Taillon BE**. Paired-end

mapping reveals extensive structural variation in the human genome. Science. 2007;318:420-6.

53. **Kotsilkov K, Popova C, Boyanova L, Setchanova L, Mitov I.** Comparison of culture method and real-time PCR for detection of putative periodontopathogenic bacteria in deep periodontal pockets. Biotechnology & Biotechnological Equipment. 2015;29:996-1002.
54. **Kumar PS, Griffen AL, Moeschberger ML, Leys EJ.** Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J Clin Microbiol. 2005;43:3944–3955.
55. **Kundapur PP, Bhat KM, Bhat GS.** Association of Trauma from occlusion with Localized Gingival Recession in Mandibular Anterior Teeth. DRJ 2009;6:71-4.
56. **Kuramitsu HK, He XS, Lux R, Anderson MH, Shi WY**. Interspecies interactions within oral microbial communities. Microbiol Mol Biol Rev 2007;71:653–670
57. **Lamont RJ, Hajishengallis G.** Polymicrobial synergy and dysbiosis in inflammatory disease. Trends in molecular medicine. 2015;21:172-83.
58. **Lang NP, Loe H.** The relationship between the width of keratinized gingiva and gingival health. J Periodontol 1972;43:623–627.

59. **Li K, Bihan M, Methé BA**. Analyses of the stability and core taxonomic memberships of the human microbiome. PloS one. 2013;8:e63139.

60. **Li Y, He J, He Z, Zhou Y, Yuan M, Xu X, Sun F, Liu C, Li J, Xie W, Deng Y**. Phylogenetic and functional gene structure shifts of the oral microbiomes in periodontitis patients. The ISME journal. 2014;8:1879-91.

61. **Lif Holgerson P, Harnevik L, Hernell O, Tanner AC, Johansson I.** Mode of birth delivery affects oral microbiota in infants. Journal of dental research. 2011;90:1183-8.

62. **Listgarten MA**. The structure of dental plaque. Periodontology 2000. 1994;5:52-65.

63. **Liu B, Faller L, Klitgord N, et** al. Deep sequencing of the oral microbiome reveals signatures of periodontal disease. PLoS One. 2012;7(6):e37919

64. **Liu WT, Marsh TL, Cheng H, Forney LJ**. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 1997;63:4516–4522.

65. **Löe H, Theilade E, Jensen SB.** Experimental gingivitis in man. Journal of periodontology. 1965;36:177-87.

66. **Loesche WJ**. Chemotherapy of dental plaque infections.. Oral Sci Rev. 1976;9:65-107.

67. **Loesche WJ. Syed SA. Stoll J.** Trypsin-like activity in subgingival plaque. A diagnostic marker for spirochetes and periodontal disease? Journal of Periodontology. 1997;58:266-73

68. **Marcy Y, Ouverney C, Bik EM, Lösekann T, Ivanova N, Martin HG, Szeto E, Platt D, Hugenholtz P, Relman DA, Quake SR**. Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proceedings of the National Academy of Sciences. 2007 Jul 17;104(29):11889-94.

69. **Mardis ER.** Next-generation DNA sequencing methods. Annu. Rev. Genomics Hum. Genet.. 2008;9:387-402.

70. **Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB**. Genome sequencing in microfabricated high-density picolitre reactors. Nature. 2005;437:376-80.

71. **Marsh PD, Moter A, Devine DA**. Dental plaque biofilms: communities, conflict and control. Periodontology 2000 2011;55:16-35.

72. **Marsh PD**. Microbial ecology of dental plaque and its significance in health and disease. Advances in dental research. 1994;8:263-71.

73. **Mason MR, Preshaw PM, Nagaraja HN, Dabdoub SM, Rahman A, Kumar PS.** The subgingival microbiome of clinically healthy current and never smokers. The ISME journal. 2015;9:268-72.

74. **Merijohn GK**. Management and prevention of gingival recession. Periodontology 2000. 2016;7:228-42.

75. **Meuric V, Le Gall-David S, Boyer E, Acuña-Amador L, Martin B, Fong SB, Barloy-Hubler F, Bonnaure-Mallet M.** Signature of microbial dysbiosis in periodontitis. Applied and Environmental Microbiology. 2017:AEM-00462.

76. **Miller WD.** The micro-organisms of the human mouth: the local and general diseases which are caused by them. S. Karger; 1890.

77. **Mitchell DF, Johnson M**. The nature of the gingival plaque in the hamster—production, prevention, and removal. Journal of dental research. 1956;35:651-5.

78. **Miyasato M, Crigger M, Egelberg J.** Gingival condition in areas of minimal and appreciable width of keratinized gingiva. J Clin Periodontol 1977: 4: 200–209.

79. **Moore WE, Moore LV**. The bacteria of periodontal diseases. Periodontology 2000. 1994;5:66-77.

80. **Morales DK, Hogan DA.** Candida albicans interactions with bacteria in the context of human health and disease. PLoS pathogens. 2010;29;6:e1000886.

81. **Mullis KB, Faloona FA**. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in enzymology. 1987;155:335-50.

82. **Newman MG, Socransky SS, Savitt ED, Propas DA, Crawford A.** Studies of the microbiology of periodontosis. Journal of periodontology. 1976;47:373-9.

83. **Nishihara T, Koseki T.** Microbial etiology of periodontitis. Periodontology 2000. 2004;36:14-26.

84. **Page RC, Lantz MS, Darveau R, Jeffcoat M, Mancl L, Houston L, Braham P, Persson GR**. Immunization of Macaca fascicularis against experimental periodontitis using a vaccine containing cysteine proteases purified from Porphyromonas gingivalis. Molecular Oral Microbiology. 2007;22:162-8.

85. **Page RC, Sturdivant EC**. Noninflammatory destructive periodontal disease (NDPD). Periodontol 2000. 2002;30:24–39.

86. **Palmer Jr RJ.** Composition and development of oral bacterial communities. Periodontology 2000. 2014;64:20-39.

87. **Paster BJ, Olsen I, Aas JA, Dewhirst FE**. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontology 2000. 2006;42:80-7.

88. **Payungporn S, Arirachakaran P, Poomipak W, Praianantathavorn K, Charalampakis G, Poovorawan Y**. Identification of Bacteria Associated with a Periodontal Disease in Thai Patients Based on Next-Generation Sequencing. Jundishapur Journal of Microbiology. 2017;10.

89. **Petersen C, Round JL**. Defining dysbiosis and its influence on host immunity and disease. Cellular microbiology. 2014;16(7):1024-33.

90. **Popova C, Dosseva-Panova V, Panov V**. Microbiology of periodontal diseases. A review. Biotechnology & Biotechnological Equipment. 2013;27:3754-9.

91. **Pozhitkov AE, Beikler T, Flemmig T, Noble PA.** High- throughput methods for analysis of the human oral microbiome. Periodontology 2000. 2011;55:70-86.

92. **Ravipudi S, Appukuttan D, Prakash PS, Victor DJ.** Gingival Recession: Short Literature Review on Etiology, Classifications and Various Treatment Options. J. Pharm. Sci. & Res.2017; 215-220

93. **Rizzo JM, Buck MJ**. Key principles and clinical applications of" next-generation" DNA sequencing. Cancer prevention research. 2012 May Cancer Prev Res (Phila) 5:887-900.

94. **Roberts FA, Darveau RP**. Microbial protection and virulence in periodontal tissue as a function of polymicrobial communities: symbiosis and dysbiosis. Periodontology 2000. 2015;69:18-27.

95. **Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, Leamon JH, Johnson K, Milgrew MJ, Edwards M,:** An integrated semiconductor device enabling non-optical genome sequencing. Nature 2011;475(7356):348-352.

96. **Saini R, Giri PA, Saini S, Saini SR**. Dental Plaque: A Complex Biofilm. Pravara Medical Review. 2015;7.

97. **Samaranayake L**. Essential Microbiology for Dentistry E-Book. Elsevier Health Sciences; 2011.

98. **Sanders WE, Sanders CC.** Modification of normal flora by antibiotics: effects on individuals and the environment. In: Koot RK, Sande MA, editors. New dimensions in antimicrobial chemotherapy. New York: Churchill Livingstone, 1984:217–241.

99. **Santigli E, Trajanoski S, Eberhard K, Klug B.** Sampling modification effects in the subgingival microbiome profile of healthy children. Frontiers in microbiology. 2017;7:2142.

100. **Sarfati A, Bourgeois D, Katsahian S, Mora F, Bouchard P.** Risk assessment for buccal gingival recession defects in an adult population. J Periodontol 2010;81:1419–1425

101. **Serino G, Wennstrom J, Lindhe J, Eneroth L**. The prevalence and distribution of gingival recession in subjects with a high standard of oral hygiene. J Clin Periodontol 1994;21:57–63.

102. **Shi B, Chang M, Martin J, Mitreva M, Lux R, Klokkevold P, Sodergren E, Weinstock GM, Haake SK, Li H.** Dynamic changes in the subgingival microbiome and their potential for diagnosis and prognosis of periodontitis. MBio. 2015;6:e01926-14.

103. **Siqueira JF, Rôças IN**. The oral microbiota: general overview, taxonomy, and nucleic acid techniques. Oral Biology: Molecular Techniques and Applications. 2010:55-69.

104. **Slots J**. The predominant cultivable organisms in juvenile periodontitis. European Journal of Oral Sciences. 1976;84:1-10.

105. **Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL**. Microbial complexes in subgingival plaque. Journal of clinical periodontology. 1998;25:134-44.

106. **Socransky SS, Haffajee AD.** Evidence of bacterial etiology: a historical perspective. Periodontology 2000. 1994;5:7-25.

107. **Socransky SS, Haffajee AD.** Periodontal microbial ecology. Periodontology 2000. 2005;38:135-87.

108. **Socransky SS, Haffajee AD**. The bacterial etiology of destructive periodontal disease: current concepts. Journal of periodontology. 1992;63:322-31.

109. **Sonnenburg JL, Fischbach MA**. Community health care: therapeutic opportunities in the human microbiome. Science translational medicine. 2011;3:78-120.

110. **Spratt DA.** Significance of bacterial identification by molecular biology methods. Endodontic Topics. 2004;9:5-14.

111. **Stout MJ, Conlon B, Landeau M, Lee I, Bower C, Zhao Q, Roehl KA, Nelson DM, Macones GA, Mysorekar IU**. Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. American journal of obstetrics and gynecology. 2013;208:226.

112. **Tanner A, Maiden MF, Paster BJ, Dewhirst FE**. The impact of 16S ribosomal RNA- based phylogeny on the taxonomy of oral bacteria. Periodontology 2000. 1994;5(1):26-51.

113. **Tanner AC, Paster BJ, Lu SC, Kanasi E, Kent Jr R, Van Dyke T, Sonis ST.** Subgingival and tongue microbiota during early periodontitis. Journal of dental research. 2006;85:318-23.

114. **Tatakis DN, Kumar PS.** Etiology and pathogenesis of periodontal diseases. Dental Clinics. 2005;49:491-516.

115. **Teles R, Sakellari D, Teles F, Konstantinidis A, Kent R, Socransky S, Haffajee A**. Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. Journal of periodontology. 2010;81:89-98.

116. **The American Academy of Periodontology.** Guidelines for Periodontal Therapy (Position Paper). J Periodontol 1998;69:396-399

117. **Trajanoski S, Klug B, Klymiuk I, Bozic M, Grube M, Wimmer G, Santigli E.** Next-generation sequencing in microbiome analysis: factors affecting reproducibility of repeated biofilm sampling of the gingival sulcus of children. Journal of Dental, Oral and Craniofacial Epidemiology. 2013;1:34-44.

118. **Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett C, Knight R, Gordon JI**. The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature. 2007;449(7164):804.

119. **Van der Velden U, Abbas F, Armand S, et al.** Java project on periodontal diseases. The natural development of periodontitis: Risk factors, risk predictors and risk determinants. J Clin Periodontol 2006; 33:540-548.

120. **Vartoukian SR, Palmer RM, Wade WG.** The division "Synergistes". Anaerobe. 2007;13:99-106.

121. **Voelkerding KV, Dames SA, Durtschi JD.** Next-generation sequencing: from basic research to diagnostics. Clinical chemistry. 2009;55:641-58.

122. **Wade WG.** The oral microbiome in health and disease. Pharmacological research. 2013;69:137-43.

123. **Waerhaug, J**. The gingival pocket. Anatomy, pathology, deepening and elimination. Odontologisk Tidskrift 1952; 60.

124. **Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, Zhao F**. Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease. Scientific reports. 2013;3:1843.

125. **Wennstrom JL**. Mucogingival therapy. Ann Periodontol 1996;1:671-701.

126. **Wiebe CB, Putnins EE**. The periodontal disease classification system of the American Academy of Periodontology-an update. Journal-Canadian Dental Association. 2000;66:594-9.

127. **Wolff, L.F.,** Natural distribution of 5 bacteria associated with periodontal disease. J Clin Periodontol, 1993;20:699-706.

128. **Xie G, Chain PS, Lo CC, Liu KL, Gans J, Merritt J, Qi F.** Community and gene composition of a human dental plaque microbiota obtained by metagenomic sequencing. Molecular oral microbiology. 2010;25:391-405.

129. **Ximenez-Fyvie, L.A., A.D. Haffajee, and S.S.** Socransky, Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol, 2000;27:648-57

130. **Yang B, Wang Y, Qian PY.** Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC bioinformatics. 2016;17:135.

131. **Zambon JJ**. Periodontal diseases: Microbial factors. Ann Periodontol 1996;1:879-925.

132. **Zaura E, Keijser BJ, Huse SM, Crielaard W**. Defining the healthy core microbiome" of oral microbial communities. BMC microbiology. 2009;9:259.

133. **Zaura E, Nicu EA, Krom BP, Keijser BJ**. Acquiring and maintaining a normal oral microbiome: current perspective. Frontiers in cellular and infection microbiology. 2014;4.

134. **Zheng , Maria Tsompana, Angela Ruscitto, Ashu Sharma, Robert Genco, Yijun Sun,and Michael J.** Buck, An accurate and efficient

experimental approach for characterization of the complex oral microbiota Microbiome. 2015;3:48.

135. **Zheng H, Xu L, Wang Z, Li L, Zhang J, Zhang Q, Chen T, Lin J, Chen F.** Subgingival microbiome in patients with healthy and ailing dental implants. Scientific reports. 2015;16:5.

136. **Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T, Podar M, Warner B, Tarr PI, Nelson DE, Fortenberry JD.** Biogeography of the ecosystems of the healthy human body. Genome biology. 2013;14:R1.

Annexures

ANNEXURE-I

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA
Tele : (044) 24530002, 24530003 - 06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOM SO EVER IT MAY CONCERN

Date: 20.12.2017

Place: Chennai

From

The Institutional Review Board

Ragas Dental College & Hospital

Uthandi,

Chennai- 600119.

The dissertation topic titled "**IDENTIFICATION OF SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVAL RECESSION USING NEXT GENERATION SEQUENCING TECHNOLOGY**" submitted by **Dr. ANISHA DEBORAH JEYAKUMAR** has been approved by the Institutional Ethics Board of Ragas Dental College and Hospital.

DR. N.S.AZHAGARASAN, MDS.,

Member Secretary,

Institutional Ethics Board,

Ragas Dental College & Hospital

Uthandi,

Chennai- 600119.

RAGAS DENTAL COLLEGE * Chennai

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI-600 119.

ANNEXURE - II

URKUND

Urkund Analysis Result

Analysed Document: doc for analysis.docx (D34901149)
Submitted: 1/22/2018 7:32:00 AM
Submitted By: anishadeborah@gmail.com
Significance: 3 %

Sources included in the report:

final thesis plagiarism11111.docx (D34429290)

Instances where selected sources appear:

13

ANNEXURE - III

CONSENT FORM

I ……S/o, w/o, d/o……
aged about …….years, Hindu/Christian/Muslim …… …….residing at ……do solemnly
And state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for

……
……

I was examined by Dr…….. and I was requested to do the following

1. Full mouth Plaque Score

2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in ……(language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorise the Doctor to proceed with further treatment or any other suitable alternative method for the study,

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu?.....) before me

Signature of the Doctor